IMPACT OF DIET COMPOSITION ON RUMEN BACTERIAL PHYLOGENETICS

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ABSTRACT

Two experiments were conducted to determine the effects of various forage to concentrate ratios on the rumen microbial ecosystem and rumen fermentation parameters using culture-independent methods. In the first experiment, cattle were fed either a high concentrate (HC) or a high concentrate without forage (HCNF) diet. Comparison of rumen fermentation parameters between these two diets showed that duration of time spent below pH 5.2 and rumen osmolality were higher for HCNF. Calculations using Simpson's index showed a greater diversity of dominant species for HCNF than in HC based on 16S rRNA PCR-DGGE. Real-time real-time PCR showed populations of *Fibrobacter succinogenes* (P=0.01) were lower in HCNF than HC diets. *Ruminococcus* spp.*, F. succinogenes* and *Selenomonas ruminantium* were present at higher ($P \leq 0.05$) concentrations in solid than in liquid digesta in both diets. The second experiment compared cattle as they adapted from a strictly forage to a concentrate diet, after which they were subject to an acidotic challenge and a recovery period (Forage, Mixed Forage, High Grain, Acidosis and Recovery). A total of 153,621 high-quality bacterial sequences were obtained from biopsied rumen epithelium, and 407,373 sequences from the solid and liquid phases of rumen contents. Only 14 epithelial genera representing >1.0% of the epimural population differed ($P \leq 0.05$) among dietary treatments. However, clustering showed a closer relation in bacterial profiles for the Forage and Mixed Forage diets as compared to the High Grain, Acidosis and Recovery diets. Several epithelial identified genera including *Atopobium, Desulfocurvus*, *Fervidicola*, *Lactobacillus* and *Olsenella* increased as a result of acidosis. However, any changes in bacterial populations during the acidosis challenge were not sustained during the recovery period. This indicates a high level of stability within the rumen epimural community. An epithelial core microbiome was determined which explained 21% of the enumerable rumen population across all treatment samples. Cluster analysis of the solid and liquid phase rumen

bacterial showed that these populations differed ($P \leq 0.10$) between forage and grain-based diets. Rumen core microbiome analysis found 32 OTU's representing 10 distinct bacterial taxa in whole rumen contents for all dietary treatments. Heifers that developed clinical acidosis vs the subclinical acidosis showed increases in the genera *Acetitomaculum, Lactobacillus, Prevotella,* and *Streptococcus*. Variation in microbial taxa as an effect of both treatment and animal was evident in the solid and liquid fractions of the rumen digesta. However, impacts of a dietary treatment were transient and despite an acidotic challenge, rumen microbiota were able to recover within a week of perturbation. The bacterial populations in the rumen are highly diverse as indicated by DGGE analysis and showed clear distinction between not only dietary treatments, individual animals, but also between epithelial, liquid and solid associated populations on the same diet. Molecular techniques provide an increased understanding of the impact of dietary change on the nature of rumen bacterial populations and conclusions derived using these techniques may not match those previously derived using traditional laboratory culturing techniques.

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DEDICATION

To the most amazing little boy I know. Finn, you are my biggest accomplishment.

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ABBREVIATIONS

ADG	Average daily gain
DDGS	Dried distillers grains with solubles
DGGE	Denaturing gradient gel electrophoresis
DMI	Dry matter intake
HC	High concentrate, ration containing minimal forage (90:10)
HCNF	High concentrate ration containing no forage
LRCpH	Lethbridge Research Centre Ruminal pH Measurement System
NDF	Neutral detergent fiber
peNDF	Physically effective neutral detergent fiber
real-time PCR	Quantitative polymerase chain reaction
SARA	Sub-acute ruminal acidosis
VFA	Volatile fatty acids

CHAPTER 1
1.0 GENERAL INTRODUCTION

The rumen microbial ecosystem is one of the most complex, diverse, and well-studied microbiological environments. Robert E. Hungate, the father of rumen microbiology, began investigating this system in the 1940's and half a decade later many of his discoveries still hold true (Hungate 1966; Krause and Russell 1996). The bacteria of the rumen have co-evolved with the host and produce the enzymes required to digest plant cell walls, enabling the host to derive energy from an otherwise indigestible feed source. The health and productivity of the ruminant is highly dependent on the rumen ecosystem (Russell 2002; Welkie *et al.* 2010), which in turn is highly responsive to changes in diet (Kocherginskaya *et al.* 2001; Li *et al.* 2009). Therefore, in healthy growing cattle, diet composition is the most important driver of hierarchical structural changes in bacterial populations (Welkie *et al.* 2010). Within the rumen ecosystem bacterial populations have been described in terms of independent yet interrelated compartments consisting of epithelial, free-living liquid, and particle-associated biofilm communities (Cheng and Wallace, 1979; McCowan *et al.* 1978). Early studies, using electron microscopy and culture-dependent methods identified a unique epithelial-associated bacterial community with specialized functions including urea hydrolysis, oxygen savaging and recycling of epithelial tissue. However, culture-dependent research is limited in its ability to describe the members of the rumen microbial ecosystem due to the fastidious nature of many of the members of this anaerobic environment (Hungate 1966; Cheng and McAllister 1997; Flint 1997). Regardless of the limitations of classical microbiology, previous literature provides a rich functional framework from within which it is possible to study rumen microbiology. The development of

molecular techniques to investigate ecological microbial communities has provided the microbiologist with a vast array of new techniques to investigate the digestive microbiota (White *et al.* 1999; Kocherginskaya *et al.* 2001; Yu and Morrison 2004; Sadet *et al.* 2007; Dowd *et al.* 2008b). The application of these techniques to the rumen under a variety of diets, through dietary transitions and under extreme ruminal pH conditions will help provide details that are key to researchers understanding the rumen microbial ecosystem and therefore ways to monitor and regulate digestive disturbances such as acidosis.

However, it is important to understand that there are inherent limitations to these culture-independent techniques which make it difficult to clearly assess the rumen microbiota to the species level, and despite the ability to demonstrate that there are a number of additional uncultured bacteria in the rumen, they remain largely uncharacterized (Krause *et al.* 2006; Deng *et al.* 2007). This leaves an opening for future collaborative work between classical culturing and more modern molecular techniques to identify and characterize these unknown species. Projects such as the Hungate 1000 (http://www.hungate1000.org.nz/), which aims to produce a reference set of 1,000 rumen microbial genome sequences, along with the continual development of next generation sequencing will continue to advance our knowledge of the rumen ecosystem, documenting not only its extraordinary diversity, but also the key genes that confer the unique ability of this ecosystem to readily convert plant cell wall carbohydrates into usable energy for the host (Cho *et al.* 2006; Khafipour *et al.* 2009; Chen *et al.* 2011; Hess *et al.* 2011; Qi *et al.* 2011).

The objectives of this literature review are to provide an overview of classical rumen microbial ecology and the current understanding of the role of rumen bacterial in the digestion of a variety of feedstuffs, under various dietary regimes as well as the role these microbes have in

digestive disturbances such as subacute ruminal acidosis (SARA). Additionally, this review will highlight the recent advances made in the field of rumen molecular microbiology and how these techniques are being currently applied to answer rumen microbiology questions from half a decade ago: what bacteria are in the rumen and what is their role in rumen digestive function?

CHAPTER 2
2.0 LITERATURE REVIEW

2.1 Microbial Ecology in the Rumen

The rumen microbial ecosystem is a complex consortium of microorganisms, which through synergy convert structural and non-structural carbohydrates into volatile fatty acids (VFA) and microbial protein for the ruminant host (Kamra 2005; Qi *et al.* 2011). This distinctive digestive habitat not only provides fairly constant conditions of moisture, pH, temperature, anaerobiosis and nutrients, but it also differs significantly from other gut environments in that there are no host mediated immune defense mechanisms, which can limit the types of organisms which can survive (Hungate 1966).

The unique ability of ruminants to utilize a wide variety of feeds is due to the highly diverse rumen ecosystem, consisting of bacteria, archaea and eukaryotes (Qi *et al.* 2011). It is estimated that of these microorganisms, bacteria account for the largest proportion of the microbiota with 1 x 10^{10}–10^{11} cells/ml, representing more than 50 genera. Ciliate protozoa (10^4–10^6 cells/ml) are represented by ~ 25 genera and 6 genera of anaerobic fungi (10^3–10^5 zoospores/ml) have also been identified (Oldham 1988; Stewart *et al.* 1997; Nicolson *et al.* 2005). These numbers however are an estimate and may underrepresent the true diversity of the rumen ecosystem as the majority of bacterial species are non-culturable (Kamra 2005). Most of our knowledge regarding rumen bacterial populations has been derived from scanning and transmission electron micrographs of microbes within the various compartments (i.e., rumen epithelium, fluid, feed particle) of the rumen (Cheng and McAllister 1997; Dehority 2003). The steady supply of substrate and continuous removal of fermentation products results in an extremely dense and diverse bacterial population in rumen fluid (Hungate, 1966). Within this population,

compartmentalization has been shown to be related to nutrient acquisition. Rumen fluid contains very large amounts of particulate material which certain bacteria adhere to and colonize (Cheng *et al.* 1977) in the process of its digestion (Figure 1). Within the particle associated rumen bacteria population there is a distinctive separation of those bacterial which are loosely-associated to the feed particles and those that are particle-associated (Cheng and Wallace 1979). Distinct adhesion mechanisms for plant cell walls and starch in various bacteria, creates a subpopulation of rumen bacteria whose specific adhesion to particulate digesta, links them to the slow-moving feed component (Cheng *et al.* 1977). There are many non-adherent species of bacteria in rumen fluid, some are found as single cells and some form slime-enclosed micro colonies (Cheng *et al.* 1976; Figure 2.1). Yet another compartment within the rumen is the bacterial colonization of the luminal surface of the stratified squamous epithelium. Extensive examination of the rumen has shown that this epimural community is morphologically heterogeneous and distinct from those bacteria associated with the liquid and solid fractions of rumen contents (Cheng *et al.* 1979).

Synergism and antagonism among physiologically different microbes, even among different genera of the same family, is so variable and complicated that it is difficult to completely quantify the role played by any particular group of microbes in the rumen (Kamra 2005; Firkins and Yu 2006). However, it is understood that microbes survive and compete in the rumen under a variety of physiological, environmental and feed associated constraints, such as limited nutrient supply in ruminants limit fed once or twice daily (Kamra 2005). Research over 50 years has repeatedly shown that rumen microbes are sensitive to changes in environment, diet, and host health (Krogh 1961; Hungate 1966; Latham *et al.* 1971; Satter and Slyter 1974; Dinsdale *et al.* 1980; Allen and Mertens 1988; Tajima *et al.* 2001; Fernando *et al.* 2010; Kong *et al.* 2010b;

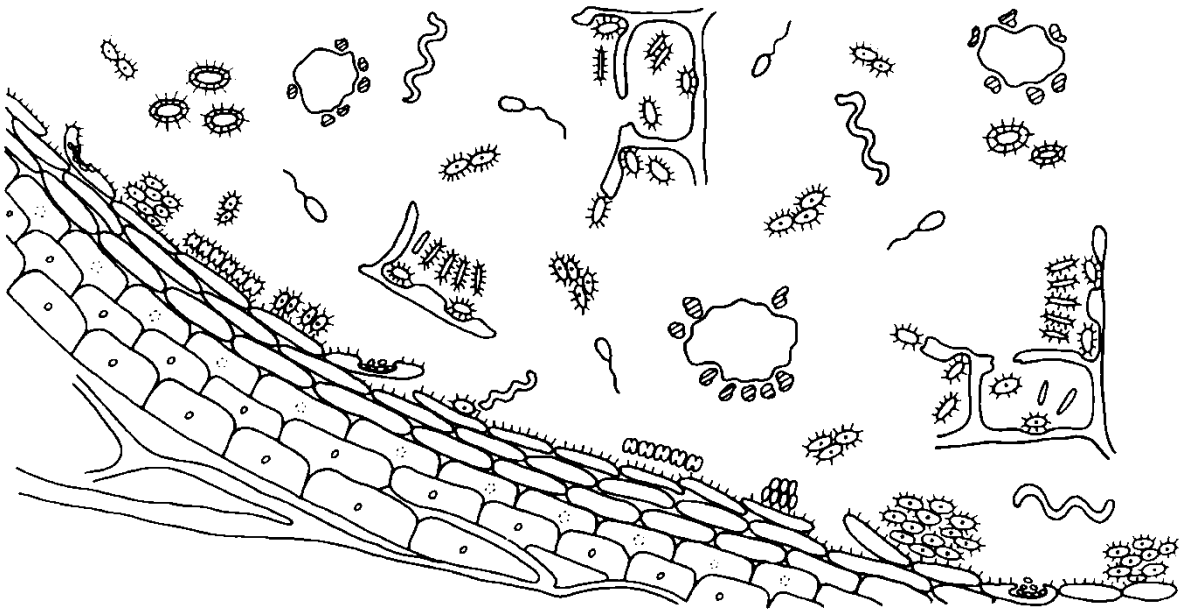

Figure 2.1. Pictorial representation of the compartmentalization of bacteria in the rumen between the epithelial adherent, particle adherent and fluid associated subpopulations. Cheng and Wallace (1979).

Sadet-Bourgeteau *et al.* 2010; Hook *et al.* 2011). However, the study of the dynamics of rumen microbial ecology has been previously limited in this unique ecosystem. The lack of truly selective media, unique end products and fastidious microorganisms has made identification of some rumen microbial species impossible with classic culture techniques (Hungate 1966; Russell and Rychlik 2001).

The use of molecular methods has renewed interest in identifying the role of various members of the microbial community in varying ruminal environments with the goal of manipulating rumen fermentation to improve feed conversion efficiency (Bergen and Bates, 1984; Eugène *et al.* 2004), decrease methane emissions (Hegarty 1999), reduce nitrogen excretion (Koenig *et al.* 2000) and prevent shedding of pathogens in feces (Callaway *et al.* 2004). Understanding which physiological and feed associated limitations have the greatest impact on rumen microbial diversity and proliferation could provide new insights into optimizing rumen health and the efficiency of meat and milk production (Stewart *et al.* 1997; Firkins *et al.* 2008).

2.2 Role of Microbes in Digestion

As a whole, rumen microbial populations have a remarkable diversity in their metabolic capability including hydrolysis of cellulose, xylans, proteins, fatty acids and fermentation of sugars (Nocek and Russell 1988; Tajima *et al.* 2001). However, a single microbial species is incapable of producing the various enzymes required to breakdown the complex plant tissues consumed by ruminants (McAllister *et al.* 1994). Digestive processes are instead accomplished by a myriad of physiologically complementary organisms that form a complex microbial consortium on the surface of plant tissues, throughout the fluid phase and on the rumen epithelium (Cheng *et al.* 1991a; McAllister *et al.* 1994).

For ruminants, being herbivores, carbohydrates are the most abundant substrate for rumen bacteria (Hungate 1975). Carbohydrate degradation in the ruminant revolves around the functional capacity of rumen microbes to ferment cellulolytic (structural carbohydrate) and non-cellulolytic (non-structural carbohydrate) feedstuffs. The relative availability of these substrates has been shown to impact both the diversity and abundance of microbes in the rumen (Tajima *et al.* 2001; Krause 2003; Sun *et al.* 2008; Kong *et al.* 2010a; Sadet-Bourgeteau *et al.* 2010; Chen *et al.* 2011). The physiological functions of the multitude of bacteria within the rumen microbiome combine to form intertwined pathways, and create an intricate balance, which is essential to the health and productivity of the host and the microbes themselves.

2.2.1 Forage Diets

Early observations on the rumen bacteria of cattle and sheep indicated fluctuations in the numbers and kinds of bacteria when different diets were fed (Bryant and Burkey 1953). Original investigations of the rumen microbiome used microscopic methods to look at the patterns of degradation on predominant feedstuffs. However, these microscopic-based studies were limited in scope because little information could be obtained on the numbers or kinds of bacteria present (El-Shazly *et al.* 1961; Dehority *et al.* 1962; Dehority and Scott, 1967; Akin 1976). Throughout the history of rumen microbiology however, it has become abundantly clear that diet composition has a major impact on the microbial ecology of the rumen.

Since the primary source of energy found in forages is structural polysaccharides including cellulose, hemicellulose and pectin, it is not surprising that cellulolytic bacteria are found at the highest levels in ruminants fed forages when compared to concentrate based diets. Previously, it was generally agreed that ruminal cellulolysis is primarily carried out by three species of bacteria: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*

(Hungate, 1966; Dehority, 1993; Flint 2008; Table 2.1). However, developments in rumen microbial ecology based on sequencing analysis have suggested that there may be more bacteria than just these three involved in the cellulolytic process (Brulc *et al.* 2009; Qi *et al.* 2011). The major constraint to degradation and utilization of intact plant cell wall polysaccharides by ruminal microbes seems to be substrate inaccessibility (Dehority, 1991). The complex cell wall matrix differs considerably among various plant species as well as with plant maturity, resulting in substantial variability in the extent to which cellulolytic bacteria can attach to and degrade cell wall polymers (El-Shazly *et al.* 1961; Akin and Barton, 1983; Chesson *et al.* , 1986; Theander, 1989). As *F. succinogenes, R. albus*, and *R. flavefaciens* specifically participate in the degradation of plant cell walls, the relative abundance of these species in forage-fed ruminants can be high. Research has shown that adherent microbial populations are numerically predominant and can accounted for up to 70 – 80 % of the total microbial population (Craig *et al.* 1987; Sun *et al.* 2008) and produce as much as 80% of total rumen endoglucanase activity (Minato *et al.* 1966; Sun *et al.* 2008). Due to the structural nature of plant cell walls, cellulolytic microorganisms must work as a consortium to produce the enzymatic profile necessary to degrade xylans, mannans, and pectins. This then allows access to the cellulose fibrils embedded within the plant cell wall (Coen and Dehority 1970; Flint *et al.* 2008). Cellulolytic bacteria typically attach directly to the fiber particles in a highly ordered fashion and form biofilms (McAllister *et al.* 1994; Flint *et al.* 2008; Krause *et al.* 2003). They are also known to be strict anaerobes, have a narrow optimal pH range, and require branched chain VFAs produced by other bacteria to grow (Hobson and Stewart 1997).

Table 2.1. The characteristics of predominant cultured ruminal microbiota adapted from (Russell and Rychlik 2001).

Species	Ruminal Substrate	Fermentation Products
Fibrobacter succinogenes	CU	S, F,A
Ruminococcus albus	CU, HC	A, F, E, H_2
Ruminococcus flavefaciens	CU, HC	S, F, A, H_2
Eubacterium ruminantium	HC, DX, SU	A, F, B, L
Ruminobacter amylophilus	ST	S, F, A, E
Streptococcus bovis	ST, SU	L, A, F, E
Succinomonas amylolytica	ST	S, A, P
Prevotella albensis	ST, PC, XY, SU	S, A, F, P
Prevotella brevis	ST, PC, XY, SU AA	S, A, F, P
Prevotella bryantii	ST, PC, XY, SU	S, A, F, P
Prevotella ruminocola	ST, PC, XY, SU	S, A, F, P
Butyrivibrio fibrisolvens	ST, CU, HC, PC, SU	B, F, A, H_2
Selenomonas ruminantium	ST, DX, SU, L, S	L, A, P, B, F, H_2
Megasphaera elsdenii	L, SU	P, A, B, Br, H_2
Lachnospira multiparus	PC, SU	L, A, F, H_2
Succinovibrio dextrinosolvens	PC, DX, SU	S, A, F, L
Anaerovibrio lipolytica	GL, SU	A, S, P
Peptostreptococcus anaerobius	AA	Br, A
Clostridium aminophilum	AA	A, B
Clostridium sticklandii	AA	A, Br, B, P
Wolinella succinogenes	OA, H_2, F	S
Methanobrevibacter ruminantium	H_2, CO_2, F	CH_4

Abbreviations are as follows; CU, cellulose; HC, hemicellulose, DX, dextrins; SU, sugars; ST, starch; PC, pectin; XY, xylans; L, lactate; S, succinate; GL, glycerol, AA, amino acids; OA, organic acids; H_2, hydrogen; F, formate; CO_2, carbon dioxide; A, acetate; E, ethanol; B, butyrate; L, lactate; P, propionate; Br, branched chain fatty acids, CH_4, methane.

Since these early studies, the development and use of anaerobic culture techniques and more recently, molecular DNA methods, have helped to identify and enumerate numerous fibrolytic microbial species. Other species, such as *Prevotella ruminocola*, *Butyrivibrio fibrisolvens* and *Lachnospira multiparus* which specialize in the digestion of hemicellulose and pectin, have also been identified as members of the cellulolytic rumen consortia in forage fed ruminants (Dehority 2003). The use of qualitative and quantitative molecular methods such as cloning and quantitative polymerase chain reaction (real-time PCR), respectively, have shown that despite the pivotal role of the major cellulolytic bacteria in ruminal fermentation of forage diets, these bacteria do not account for the majority of the forage-fed microbiome. *Prevotella ruminicola* has been found to account for as much as 14 – 22% of the total bacterial population (Tajima *et al.* 2001; Kong *et al.* 2010) compared with much lower relative abundances of *Ruminococcus* (1.6%) and *Fibrobacter* spp. (0.1 – 16%; Kong *et al.* 2010).

2.2.2 Mixed Forage-Concentrate Diets

Increases in intensive animal agriculture and growth of international trade have resulted in a more competitive market for beef and a more diverse array of feedstuffs available for use in cattle diets. These events have resulted in changes in how cattle are fed in many different production systems, from cow-calf production to the feedlot. The surplus production of distiller's grains entering the animal feed industry is one of many changes that have resulted in an increased number of cattle being fed mixed forage-concentrate diets (Al-Suwaiegh *et al.* 2002; Morris *et al.* 2005 and 2006). Improved cattle production (increased ADG, improved feed conversion), as a result of microbial growth and adaptation to increased nutrient availability resulting in increased VFA production, has driven the continued use of mixed diets in cattle feeding (Fron *et al.* 1996).

Increases in energy availability are the most common substrate changes when moving from forage-based to a mixed forage-concentrate diet. Ruminal microorganisms can ferment starch and other more readily available sugars at an increased rate and to a greater extent than fibrous substrates, resulting in an increase in the productivity of the host (Nocek and Russell, 1988; Emmanuel 2008). However, more rapid fermentation of feedstuffs results in fluctuating ruminal pH with an increase in the variability of pH (4.5-7.0) as compared to diets that contain only forage. This variation in rumen pH is influenced by the total intake of fermentable carbohydrate, the inherent capacity of the host to buffer the rumen through excretion of salivary bicarbonate, and rates of VFA utilization by the host and microbes, as well as the absorptive capacity of epithelial cells (Nagaraja and Titgemeyer 2007; Penner *et al.* 2009). Research has also shown higher ruminal bacterial numbers in response to highly digestible diets as a result of increased levels of readily fermentable carbohydrate (Krogh 1961; Mann 1970; Latham *et al.* 1971). Many species of ruminal bacteria actively ferment starch and utilize the arising intermediate products (Figure 2.2) and depending on dietary substrate, the proportion of amylolytic bacteria in the rumen can be as high as 90 to 95% of total culturable bacteria in grain-fed animals (Leedle *et al.* 1982). Ruminal bacteria have been shown to attach to starch granules (Minato and Suto, 1979; McAllister *et al.* 1990c) and to digest starch in the rumen liquid (McWethy and Hartman, 1977; Cotta, 1988). Repeated culture studies have shown that the predominant amylolytic bacteria in the rumen include species of *Butyrivibrio*, *Eubacterium*, *Lactobacillus*, *Prevotella*, *Ruminobacter*, *Selenomonas* and *Streptococcus*, (Stewart *et al.* 1997; Tajima 2000; Table 2.1).

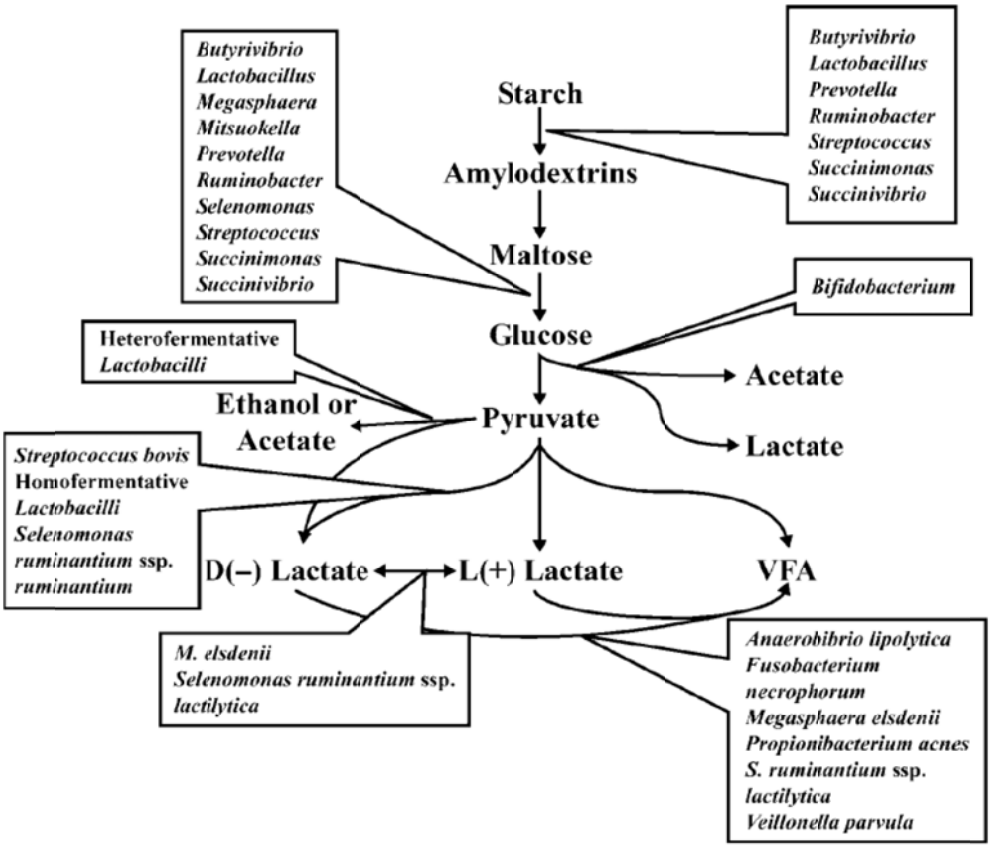

Figure 2.2. Amylolytic, maltose-fermenting, glucose-fermenting and lactic acid fermenting bacteria involved in the fermentation of starch to lactic acid and VFA in the rumen of grain-fed cattle. Adapted from Nagaraja and Titgemeyer (2007).

In addition to the role of bacteria, starch fermentation in the rumen is also impacted by the presence of protozoa (Hungate 1966). Early observations with ruminants fed high concentrate diets suggested that protozoa were practically eliminated due to rumen acidity, especially when grain was provided ad libitum (Eadie *et al.* 1970; Slyter *et al.* 1970). However, studies have shown that protozoa may be present in significant numbers, but with reduced diversity in feedlot cattle fed a variety of high grain diets (Towne *et al.* 1990). Protozoa rapidly engulf starch granules (Coleman 1974) and thus compete effectively with amylolytic bacteria for substrate (Owens *et al.* 1998). Starch consumed by protozoa is fermented at a slower rate than when colonized by amylolytic bacteria and the main products of fermentation are VFA rather than lactate. Therefore, ruminal protozoa reduce the rate and extent of starch digestion in the rumen, shifting the site of digestion to the small intestine when cattle are fed high-concentrate diets. Researchers have suggested that with high-concentrate diets, protozoa have a stabilizing effect on rumen fermentation and may play a beneficial role in the reduction of starch-induced mediated digestive disturbances such as subacute ruminal acidosis (SARA; Mackie *et al.* 1978; Mendoza *et al.* 1993; Brossard *et al.* 2004).

Those amylolytic microorganisms within the fluid phase must continually seek out soluble substrates as compared to those which are attached to starch granules (McAllister *et al.* 1994). It has been shown that for *S. bovis,* an adherent bacterium, attachment is not site-specific, whereas *R. amylophilus* preferentially attaches to the surface of starch granules and *B. fibrisolvens* both readily digest isolated starch granules and colonizes the endosperm cell wall (Cotta, 1982; McAllister *et al.* 1994). Overall affinity for starch varies for each microbe based on whether they are solid associated, liquid associated or epithelial adherent (McAllister *et al.* 1994).

Using real-time PCR, Tajima *et al.* (2001) found increased levels of *Prevotella bryantii*, and *Selenomonas ruminantium* and decreased amounts of *P. ruminicola*, *F. succinogenes*, and *R. flavefaciens* in the rumen of cattle fed mixed concentrate-forage diets compared with those fed a forage diet. Similarly, Li *et al.* (2009) determined that as dietary forage decreased, so did the relative proportions of cellulolytic species in the rumen. *Fibrobacter succinogenes* (0.3 – 1.6%) and *Ruminococcus* spp. (5.7 – 10.6%) represented a much lower percentage of the total population mixed concentrate-forage diets. However, unlike Tajima *et al.* (2001), *P. ruminicola* was not enumerated by real-time PCR in the study of Li *et al.* (2009). Enumeration (percent of total) of *Eubacterium ruminantium* (0.27 – 0.62%) *S. ruminantium* (0.3 – 1.6%), *B. fibrisolvens* (1.7 – 3.4%) and *S. bovis* (>0.1%) temporally and spatially within the rumen was only able to account for 20% of the total ruminal bacteria. Despite the evolution of techniques with which to identify and enumerate bacteria within the rumen, as much as 80 – 90% of the bacterial population has yet to be characterized.

The majority of rumen microbial ecology research has focused on the solid, liquid or entire contents of the rumen looking at qualitative and quantitative differences (Kong *et al.* 2010). However, the rumen epithelial fraction, despite comparatively minimal investigation, is believed to be a more distinctive microbial community (Cheng *et al.* 1979; Sadet-Bourgeteau *et al.* 2010). The most common difference found between the rumen contents and epimural communities is an increase in *Proteobacteria* (Cho *et al.* 2006; Larue *et al.* 2005; Tajima *et al.* 1999; Yu *et al.* 2006; Sadet-Bourgeteau *et al.* 2010). This is likely due to the presence of trace amounts of oxygen, diffusing through the rumen epithelium as many members of this Phylum are microaerophiles or facultative anaerobes and therefore not as sensitive to oxygen (Sadet-Bourgeteau *et al.* 2010). While bacteria themselves provide the majority of protein available for

uptake by the ruminant, some species are also critical in protein breakdown (McCowan *et al.* 1978), and ammonia recycling (Cheng *et al.* 1979; McCowan *et al.* 1978; Wallace *et al.* 1979). Many of these protein degrading and ammonia recycling species are believed to be part of the epimural community. While major proteolytic bacteria in rumen contents include *P. ruminicola, B. fibrisolvens*, *R. amylophilus, Selenomonas ruminantium*, and *S. bovis*, the relative proportions of these species augmenting proteolytic activity depend on the size and composition of the overall bacterial population (Wallace 1985). Early analysis of the epimural populations found predominant bacteria to include *B. fibrisolvens*, *P. ruminicola*, and *Lactobacillus* spp. (Dehority and Grubb 1981). With the development of molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing, additional ruminal bacteria have been identified across a variety of diets, including the genera *Clostridium*, *Succiniclasticum*, *Syntrophococcus*, *Ottowia*, *Campylobacter*, *Desulfobulbus*, and *Porphyromonadaceae*. Research in the solid and liquid fractions of the rumen have clearly shown that dietary changes impact the microbial populations of the rumen (Kocheriginskaya *et al.* 2001; Tajima *et al.* 2001). While recent studies have indicated that this may also be true of the rumen epimural community, the specifics of such changes are still not well understood (Sadet-Bourgeteau *et al.* 2010; Chen *et al.* 2011).

2.3 Impact of Digestive Disturbances on Ruminal Microbiome

The most profound changes in the rumen bacterial community of cattle occur during weaning and in adult animals, during dietary change such as the switch from roughage to high-grain diets, or transfer from hay/concentrate to pasture feeding (Tajima *et al.* 2000). Rapid transition from a high-forage diet to a high grain diet is common practice in the nutritional management of feedlot cattle (Fernando *et al.* 2010; Chen *et al.* 2011). By changing the feed proportions in a diet to

incorporate more concentrate and less forage, ruminal total VFA production increases, while acetate to propionate ratios and pH decrease (Schwartzkopt-Genswein *et al.* 2003; Beliveau and McKinnon 2008). These changes then impact the rumen microbial communities causing marked decreases in cellulolytic species and increases in lactic acid producing and utilizing species (Nagaraja and Titegemeyer 2007; Russell 2002). As a result of these changes in the ruminal environment, time is required to establish a stable microbial population (Bevans *et al.* 2005). The VFA composition of the rumen fluid during transition from hay to high-grain diet can be considered the same as during the development of grain overload (SARA; Whitford *et al.* 1998; Garret *et al.* 1999; Tajima *et al.* 2000). However, the physiological consequences are not the same. Total ruminal acid balance in these situations can be exacerbated by decreased rates VFA absorption and utilization due to abnormal ruminal papillae or rumenitis, reduced production of acid neutralizing saliva due to limited ruminantion, and decreased rumen motility resulting in slower passage from the rumen (Nagaraja and Titegemeyer 2007). When ruminal pH is higher than 5.5 on a high-grain/transition diet, lactic acid does not accumulate in the rumen. However, pH less than 6 inhibits many cellulolytic bacteria altering rumen fermentation (Nocek 1997; Hook *et al.* 2011). If the rumen microbiome is given time to adapt to the increased VFAs and lower pH associated with high concentrate/ highly digestible diets, adaptive responses developed by individual bacteria within biofilms may be sufficient to cope with the subtle environmental changes that occur within the rumen on a daily basis (McAllister *et al.* 1994).

Original observations of the impact of diet change on the rumen microbiota were reported by Hungate *et al.* (1952). It was noted that the amount of grain capable of inducing acute indigestion in hay-fed animals caused no ill effects if the animals were gradually accustomed to this substrate. Although the existence of lactate-utilizing bacteria has been known for some time

(Mackenzie, 1967), the relationship between grain adaptation and an increase in the population of bacteria that utilize lactate was not yet understood. Since that time, the change in the rumen bacterial populations during acidosis and during the stepwise adaptation to high-concentrate diets has been investigated in several studies (Hungate 1966; Russell and Hino 1985; Goad *et al.* 1998; Brown *et al.* 2000; Tajima 2000; Khafipour *et al.* 2009). However, the methodology for characterization of transient microbiota during both, the development of acidosis and the succession of predominant rumen bacterial phylogroups during the switch from roughage to high grain diets, has been essentially cultivation-based. It is therefore likely that important contributing microorganisms have not been detected. Since culture-based analysis provides a static snapshot of the system, no functional role can be assigned to the uncultivated groups, and key aspects of bacterial interactions may be missed. The use of molecular analysis methods (real-time PCR, clone libraries) for analyzing bacterial succession during digestive disturbances has confirmed some of the earliest findings such as disappearance of ruminococci and other fibrolytic bacteria and prevalence of lactate-producing and utilizing bacteria during acidosis (Tajima *et al.* 2000; Fernando *et al.* 2010). Fernando *et al.* (2010) documented increases in *Megasphaera elsdenii* (11x), *S. bovis* (2x)*, S. ruminantium* (30x), and *P. bryantii* (8,000x) and decreases in *F. succinogenes* (40x), *B. fibrisolvens* (20x) during adaptation to a high concentrate diet. However, the significant increases in *M. elsdenii, S. bovis,* and *P. bryantii* were all transient and lasted only during the transition phase. The use of denaturing gradient gel electrophoresis (DGGE) on epithelial samples from cattle undergoing transition from a forage to a concentrate diet was found to detect Treponema spp., *Ruminobacter* spp., and *Lachnospiraceae* spp. (Chen *et al.* 2011). Many molecular-based studies still identify a large percentage of clones with unique

sequences even though more sequence data from rumen bacteria is being deposited in microbial gene banks (Chen *et al.* 2011; Fernando *et al.* 2010; Tajima *et al.* 2000).

2.4 Rumen Microbial Component of Acidosis

Acidosis is a major concern in ruminants fed high concentrate diets (Nocek 1997; Nagaraja and Chengappa 1998; Owens *et al.* 1998; Galyean and Rivera 2003). Acidosis was defined as a metabolic disorder caused by the consumption of large amounts of ruminally-degradable carbohydrate, low amounts of effective fibre, or both (Burrin and Britton 1986; Nocek 1997; Owens *et al.* 1998; Krause and Oetzel 2006). Sub-acute ruminal acidosis is less severe, but a more difficult to manage. It manifests itself sub clinically through reductions in feed intake and performance (Slyter 1976; Krehbiel *et al.* 1995; McAllister *et al.* 1996; Owens *et al.* 1998). Furthermore, acidosis-related problems can greatly affect the profitability of feeding cattle due to the increased incidence of liver abscesses leading to condemnation, reduced weight gain, decreased feed efficiency and decreased carcass yield (Nagaraja and Chengappa 1998).

If ruminants are fed fibre-deficient diets, ruminal mixing, eructation, rumination and saliva flow decreases, fermentation acids accumulate, ruminal pH declines (Hungate 1966; Allen 1997) and acidosis develops from the proliferation of starch-fermenting and lactate-producing bacteria (i.e., *S. bovis*, *Lactobacillus* spp.; Goad *et al.* 1998; Russell and Rychlik 2001). If the dietary shift is gradual, *M. elsdenii* and *S. ruminantium* can convert lactic acid to acetate and propionate. The resulting ruminal pH is not as severely affected and the ruminal ecology is not drastically altered (Nocek 1997; Nagaraja and Titegemeyer 2007). However, even high concentrations of VFAs can cause sub-acute ruminal acidosis and pH sensitive ruminal bacteria such as those which ferment cellulose are inhibited if the ruminal pH is below 6.0 (Russell and Wilson 1996). Simultaneously, there is an increase in lactic acid concentration due to proliferation of lactate-

producing microbes (Nocek 1997; Nagaraja and Titegemeyer 2007). The growth of lactate-producing organisms occurs because they are tolerant of low pH whereas lactate-utilizing microbes are sensitive to low pH (Nocek 1997; Owens *et al.* 1998; Galyean and Rivera 2003). Variations in microbial populations can be detected at different levels of acidosis, ranging from the mild or subacute to the acute a state (Tajima 2000). These and other cultivation-based studies have demonstrated that *S. bovis* is a major culprit in clinical lactic acidosis (Tajima 2000). *S. ruminantium* and *M. elsdenii* are two lactic acid utilizing bacteria which are able to tolerate lower pH conditions have been shown to be effective in preventing acid accumulation in the rumen (Tajima 2000; Nagaraja and Lechtenberg 2007).

The ruminal epithelium is not protected by mucous, thus even brief periods of sub-acute acidosis can cause inflammation, ulceration and scarring (Owens *et al.* 1998). Lactate accumulation promotes the growth of *Fusobacterium necrophorum*, a lactate utilizing bacterium that infects ruminal ulcers and if it passes from the rumen and colonizes the liver can form abscesses (Berg and Scanlan 1982; Nagaraja and Chengappa 1998; Tadepalli *et al.* 2009). Less than 0.3% of fattening beef cattle in feedlots die from grain-related digestive problems (Smith 1998), but chronic sub-clinical acidosis plagues the cattle industry (Owens *et al.* 1998). Cattle that bloat or have sub-acute acidosis consume less feed or must be culled and ~13% of the livers are condemned owing to bacterial abscesses (Smith 1998).

2.5 Individual Animal Variation

There are a number of factors that influence the susceptibility of an animal to digestive disturbances, but the most prominent are dietary composition, eating and ruminating behaviour, total DMI and the nature of the diet change (Stone 2004; Bevans *et al.* 2005; Nagaraja and Titegemeyer, 2007). However, individual animal susceptibility to acidosis is variable and

inherent animal susceptibility to the condition is difficult to predict. The variation among individuals in their susceptibility to ruminal acidosis is a concern in production where a balance between cattle health and performance must be maintained. In order to account for the large variability in the pH and health response of cattle to an acute acidosis challenge, a large number of individuals must be tested which creates a problem when such severe challenge models are used (Nagaraja and Titgemeyer 2007). This variation has received considerable attention in recent studies investigating dietary adaptation in beef cattle (Bevans *et al.* 2005) and reviews focusing on adaptation of feedlot cattle to high grain diets (Brown *et al.* 2000; Schwartzkopf-Genswein *et al.* 2003). However, there is no clear understanding of what determines an individual animals' susceptibility to SARA. From a physiological perspective, animal variation in susceptibility to SARA has been previously researched by Penner *et al.* (2009). This group looked at the variability in the capacity of the ruminal epithelium to uptake acetate and butyrate. Data indicated that those cattle that had a greater absorptive capacity for acetate ($P = 0.024$; $r^2 = 0.212$) and butyrate ($P = 0.033$; $r^2 = 0.191$) were inherently less susceptible to SARA. While these data indicate that there is a physiological component to the susceptibility of an individual animal to SARA, there is still a large component (79-81%) of variability in VFA absorption that remains unexplained. While the variability in expression may be partially explained by host genetics, a large portion is likely due to differences in the adaptive response of the microbial population. Understanding microbial succession and host-microbe interactions in the rumen and how this influences why cattle differ in their tolerance to SARA may provide new strategies to reduce subacute and acute acidosis in ruminants.

2.6 Rumen Molecular Microbiology

Until recently, characterizing the rumen microbial ecosystem has been a difficult endeavour due to the complex nutritional and environmental requirements of the bacteria therein. Traditionally, identifying bacteria in any ecosystem was dependent on successful isolation, purification and cultivation of each individual species. However, since most rumen organisms are obligate anaerobes with only a small proportion being facultative anaerobes (Hobson and Stewart 1997), culturing them has been one of the main challenges faced by rumen microbiologists (Krause and Russell 1996). When Hungate (1966) published "The Rumen and Its Microbes", approximately 23 bacterial species were believed to be predominant in the rumen. By 1996, the number exceeded 200 (Krause and Russell 1996) and is more recently estimated to be closer to 1,000 individual species (Deng *et al.* 2008). Of the numerous individual species believed to be in the rumen, it has been presumed that only a minute fraction (<1%) of the microbes within most ecosystems can be recovered by cultivation-based techniques (Amann *et al.* 1995). Recent developments in molecular biology allow researchers to characterize uncultivable microorganisms in various ecosystems via culture-independent techniques (Whitford *et al.* 1998). Using these methods, genomic DNA can be extracted from rumen samples where bacterial genes of interest (i.e. 16S rRNA gene) can be purified, amplified, and sequenced for taxonomic identification. Each sequence is identified as a terminal node in a phylogenetic analysis and is then considered as a unique organism and labelled as an operational taxonomic unit (OTU). Edwards *et al.* (2004) analyzed rRNA libraries from published rumen library studies and public databases and identified 341 OTU's, indicating that culture-based estimates of the rumen microbiota have significantly underestimated ruminal diversity (Firkins and Yu, 2008).

The small subunit ribosomal RNA (SSU rRNA) gene is a universal gene present in all prokaryotes (16S) and eukaryotes (18S; Deng *et al.* 2008). The ubiquity, genetic stability, and high-copy number of the SSU rRNA gene makes it an ideal molecular marker to characterize microbial communities (Wright *et al.* 2004). As a result of these characteristics, the SSU rRNA has received more attention than any other gene target and subsequently a large numbers of SSU rRNA sequences have been deposited in public databases (Deng *et al.* 2008). The 16S rRNA gene is also comprised of highly conserved regions interspersed with hypervariable regions which are used to charcterize phylogenetic relationships and to taxonomically characterize organisms (Wright *et al.* 2004; Deng *et al.* 2008). By amplifying regions of the 16S rRNA gene with universal primers which are complimentary to the highly conserved regions, areas of hypervariability are also amplified and the resulting amplicons exhibit a high degree of specificity for the bacteria in a given sample (Amann *et al.* 1995).

2.6.1 Current Molecular Techniques for Ecological Characterization in the Rumen

The study of phylogenetics looks at the evolutionary relations among groups of organisms (Edwards and Cavalli-Sforza 1964). At the molecular level, these evolutionary relationships are determined using hereditary molecular differences, mainly in DNA sequences. Phylogeny is based on the fundamental principle that different species descended from common ancestors. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree (Figure 2.3). A phylogenetic tree represents a hypothesis of the order in which evolutionary events are assumed to have occurred (Edwards and Cavalli-Sforza 1964).

Figure 2.3. Example of a phylogenetic tree of prokaryotes. The phylogenetic associations of prokaryotes which are divided into two distinct Domains, the Archaea (grey) and Bacteria (black).Bootstrap values from 1000 replicates are shown when ≥ 500. (Macario *et al.* 2006).

Bacteria comprise the vast majority (>90%) of prokaryotes and an important aspect of understanding prokaryotic phylogeny is to understand the evolutionary relationships among them. Based on the 16S rRNA analysis, the bacterial domain is presently divided into 25 phyla, several of which are known to be present in the rumen (Figure 2.4). However, division within the bacterial domain is arbitrary and constantly evolving as new data are generated and there are currently no truly objective criteria for identifying main groups. However, many molecular procedures provide promise for characterising the unknown, unculturable populations of rumen microbes, particularly if used in combination with other emerging technologies such as single cell analysis (Polz *et al.* 2003; Zoetendal *et al.* 2004). Cloning and random selection of clones for sequencing of 16S rRNA genes has been recently used more frequently to describe the diversity of microbes in the rumen (Whitford *et al.* 1998; Firkins and Yu *et al.* 2003 Koike *et al.* 2003b; Larue *et al.* 2004).

Bacteroidetes (formerly termed *Cytophaga-Flexibacter-Bacteroides*) have generally been over represented by cultivation-based procedures compared with sequencing data (Tajima *et al.* 2001) with the percentage of sequences belonging to this phylum ranging from 2 to 79%. The next major phylum, Firmicutes (formerly termed Low G+C Gram-Positive Bacteria) has been found to range from 11 to 95% of the total bacterial population. Among these studies, there has been no consistent data regarding whether rumen compartmentalization (*i*.e. solid-associated or liquid associated) was related to the relative abundance of Firmicutes versus Bacteroidetes. However, within studies, Firmicutes appear to be more associated with the particulate fraction and Bacteroidetes, more with the fluid fraction (Tajima *et al.* 1999; Larue *et al.* 2004). Dietary changes have been found to influence the diversity and community composition with cellulolytic Ruminococcus and Treponema (Spirochaetes phylum) associated with forage diets, and

Figure 2.4. Bacterial phyla representing 16S rRNA gene sequences of rumen origin (Kim *et al.* 2011).

R. amylophilus related species (Proteobacteria phylum) found in cattle fed grain-based diets (Kocherginskaya *et al.* 2001; Koike *et al.* 2003). Tajima *et al.* (2000) noted increased lactate-producing and lactate-utilizing bacteria following dietary transition from a forage to a high-grain diet. Feeding only forage increased the number of sequences distantly related to current isolates of the Firmicutes, whereas feeding grain tended to increase the number of sequences that were more closely related to *Selenomonas* and *Prevotella*, with the opposite trend noted for *Ruminococcus* and *Butyrivibrio* (Tajima *et al.* 2000).

Significant variation among studies can generally be explained by variability in PCR conditions (Tajima *et al.* 2001), primers or cloning vectors used (von Wintzingerode *et al.* 1997), or differences in experimental design. An example of this can be seen in studies utilizing clone libraries, where *Ruminococcus* are generally poorly represented, and *F. succinogenes* has been virtually absent. Likely this results from poor amplification of genomic DNA from these bacteria during the early amplification cycles (Firkins and Yu, 2006) when DNA flanking the 16S rRNA interferes with amplification by the formation of inhibitory secondary structures (Firkins and Yu, 2006).

2.6.2 Fingerprinting Techniques

Rumen microbes are extremely diverse and while cloning techniques provided an accurate assessment of a bacterial community, these techniques were labour intensive and costly (Deng *et al.* 2008). So, in order to study population dynamics, genetic fingerprinting techniques are needed (Muyzer 1999). The general strategy for genetic fingerprinting of microbial communities consisted of DNA extraction, rRNA amplification and subsequent analysis of PCR products. The main fingerprinting techniques used in rumen microbial ecology include terminal restriction fragment length polymorphisms (TRFLP), denaturing gradient gel electrophoresis (DGGE) and

temperature gradient get electrophoresis (TGGE; Zoetendal *et al.* 2004; Deng *et al.* 2008). There are a number of additional methods including single strand conformation polymorphism (SSCP), random amplified polymorphic DNA (RAPD) and automated ribosomal intergenic spacer analysis (ARISA), but these approaches have been used minimally in rumen microbial ecology.

Denaturing gradient gel electrophoresis examines microbial diversity based on electrophoresis of PCR-amplified 16S rRNA fragments (Muyzer *et al.* 1993). Separation of amplicons into a profile of amplified bands is based on the decreased electrophoretic mobility of partially melted double stranded DNA molecule in polyacrylamide gel containing a linear gradient of denaturant consisting of a mixture of urea and formamide (Muyzer 1999). This technique is based on the assumption that each band within a profile represents one species. However, without additional techniques the identity of the band is unknown. Kocherginskaya *et al.* (2001) applied this procedure for the analysis of ruminal bacterial diversity and found that in relation to diet, grain fed animals displayed more diverse and rich bacterial populations than hay-fed animals. As a technique for studying microbial diversity, DGGE has been shown to have decreased bias when compared with the sequencing of clone libraries if similar numbers of bands to clones are compared from the same sample (Muyzer *et al.* 1993). Denaturing gradient gel electrophoresis allows the simultaneous analysis of multiple samples (Muzyer 1999) and depends on the melting behavior of the PCR product rather than size, making it more discriminating than T-RFLP (Moeseneder *et al.* 1999). In addition, the distinct banding pattern of DGGE is indicative of different assemblages of species, which not only allows for visualization of genetic diversity but also its quantification through biodiversity indices (Simpson *et al.* 1999; McCraken *et al.* 2001).

Studies using T-RFLP digest DNA samples with restriction enzymes to create a unique peak profile based on the position of a restriction site closest to the labeled end of an amplified gene. Automated ribosomal intergenic spacer analysis involves PCR amplification of a region of the rRNA gene operon between the small (16S) and large (23S) subunits called the intergenic spacer region. The products of this amplification are electrophoresed in a polyacrylamide gel, stained and the result is a complex banding pattern similar to that produced by DGGE. As community profiling becomes more popular for rumen bacterial community analysis (Fisher and Triplett 1999; Palmonari *et al.* 2010; Weimer *et al.* 2010; Welkie *et al.* 2010) as a rapid technique, it is clear that it is best used in conjunction with more accurate but labour-intensive methods such as 16S rRNA gene cloning and sequencing when fine-scale spatial and temporal resolution is needed (Fischer and Triplett, 1999).

As with any analysis in its infancy, there are limitations with each technique which must be acknowledged in the context of the research. Overall, DGGE profiling does not capture the full diversity of the rumen ecosystem due to the fact that only abundant populations are detected (Kocherginskaya *et al.* 2001; Klieve *et al.* 2007) and therefore additional quantitative procedures are needed to account for important populations of bacteria whose rRNA is amplified with a lower PCR efficiency using universal primers (Larue *et al.* 2005). Users must also account for gel-to-gel variation, variation in the procedure for each primer set, recovery of multiple sequences from a single band, and spill-over of bands from adjoining lanes (Deng *et al.* 2007). Community profiling by T-RFLP can overcome the gel-to-gel limitation, but the ability to trace the source of the profile difference through DNA sequencing is more challenging (Muyzer 1999; McCraken *et al.* 2001; Deng *et al.* 2007). However, ARISA uses a unique area of the gene to separate individual OTU's, decreasing the PCR bias that is prevalent with DGGE. However, a

full critique of ARISA analysis in comparison with other community profiling techniques has not been performed using the rumen as a reference. With continual development and improvement in molecular technology, including instrumentation and computer cluster analysis, these techniques offer a relatively simple and systematic approach for initial profiling and comparison.

2.6.3 Quantification Techniques

Soon after the development of rRNA-based procedures, researchers adapted them for quantitative purposes in a variety of ecosystems (Leser *et al.* 2002; Firkins and Yu 2006). Conventional PCR assays detect PCR products at the end stage of a PCR reaction, where exponential amplification is no longer being achieved (Denman and McSweeney, 2005). Therefore, this approach can result in different quantities of amplicons being generated which affects the accuracy and precision of such techniques, resulting in relatively large variability (Weimer *et al.* 1999; Krause *et al.* 1999b). Competitive PCR (cPCR) was developed to accurately quantify the initial amount of template by using a known target fragment. In cPCR, a known amount of target DNA is added to each sample and competes with the template DNA for the same set of primers. The ratio of the amounts of the two amplified products as determined after the amplification is complete and reflects the ratio of the amounts of the template DNA to competitive target DNA (Koike and Kobayashi, 2001). Comparatively, real-time PCR allows for amplified DNA to be detected as the reaction progresses. This is done by using either non-specific fluorescent dyes that intercalate with any double-stranded DNA, or more commonly, sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. Real-time PCR is therefore the most commonly used technique in DNA quantification due to its ease of use.

When using both real-time (Ouwerkerk *et al.* 2002b; Klieve *et al.* 2003) and competitive (Koike and Kobayashi 2001) PCR, the abundance of target populations present in the rumen fluid is calculated using non-linear regression after spiking serial dilutions of a standard into a constant amount of the sample (Sylvester *et al.* 2004a). Accurate quantification is also dependent on the specificity of probes or primers to their target. Specificity can be designated to individual strains, species, groups within a genus, and even larger taxonomic classifications. Despite similarities however, competitive PCR is time-consuming and thus much less feasible for routine usage in studies involving a large number of samples. Therefore real-time PCR is the more commonly employed method (Schmittgen *et al.* 2000; Ouwerkerk *et al.* 2002b).

Since the detection of bacteria is dependent on efficacious primers for targeting bacteria, the development of bacterial primers has been mostly limited to those bacteria that could be cultured and the DNA extracted for sequencing. These species include the three major cellulolytic species previously mentioned. Despite the cultivability of *F. succinogenes*, *R. flavefaciens* and *R. albus*, Krause *et al.* (1999) found their combined signal only accounted for 4% of the total bacteria.

2.6.4 Next-generation sequencing technology

Since 2005, the advent of parallelized high-throughput sequencing technologies has revolutionized research in the area of genomics and metagenomics (Zhou *et al.* 2010). In comparison to the Sanger sequencing method, which is arduous, next-generation sequencing technologies have automated the sequencing process through simultaneous amplification and sequencing of millions of DNA sequences within multiple samples (Wilhelm 2009; Zhou *et al.* 2010). The rapid development of new sequencing platforms, decreased sequencing costs, increased computational power and advancement in analytical analysis tools has vastly improved knowledge of the rumen microbial community (Zhou *et al.* 2010). The two most frequently used

platforms are the Roche 454 FLX Titanium and the Illumina Genome Analyzer (GA) II (Luo *et al.* 2012). Next generation sequencing platforms produce millions of short sequence reads, which vary in length from tens of base pairs (bp) to ~800 bp. Since, read lengths are still shorter than the average bacterial gene length (~950 bp) reads are generally assembled into longer contigs (Luo *et al.* 2012). A systematic comparison of the Roche 454 and Illumina platforms for metagenomic studies by sequencing the same community DNA sample showed that the two methods agreed on over 90% of the assembled contigs and 89% of the unassembled reads as well as on the estimated gene and genome abundance in the sample (Luo *et al.* 2012). These findings suggest that both next-generation sequencing technologies are reliable for quantitatively assessing genetic diversity within natural bacterial communities. However, Illumina, and short-read sequencing in general, may be a more appropriate method for metagenomic studies to ensure an accurate assembly of contigs without the errors associated with longer sequencing reads (Luo *et al.* 2012). Furthermore, Illumina short reads would be limited in being able to identify bacteria beyond the Phylum or Order taxa levels.

Callaway *et al.* (2010) looked at the impact of DDGS on microbial populations of the rumen and feces of cattle and found that Firmicutes: Bacteroidetes ratios were smaller in 25% vs. 50% DDGS-based diets compared with the control which corresponded with a decrease in pH in the 50% diet compared to the control. Dowd *et al.* (2008) evaluated the microbiome from the feces of 20 commercial lactating dairy cows to determine the most prevalent genera and found 43 genera, which were present in all samples. Rey *et al.* (2011) looked at the establishment of the rumen commensal bacteria in dairy calves from birth to weaning using pyrosequencing. They found 430 different taxa with the major phyla being Actinobacteria, Bacteroidetes, Fimicutes and Proteobacteria (10, 15, 39 and 31% respectively). However, this research did not include analysis

at the genus. Most recently, Jami and Mizrahi (2012) used pyrosequencing to determine the composition and similarity of bacterial communities across 16 dairy cattle in an effort to define a "core microbiome". They found 51% similarity in bacterial taxa across samples when abundance and occurrence were analyzed. Thirty-two genera were shared by all samples exhibiting high variability in abundance across samples. These data suggests that though there is considerable bacterial diversity within the rumen of different hosts, the microorganisms were phylogenetically related and it was proposed that functional requirements of the rumen ecosystem tend to select for a predominant common taxa that potentially share similar genetic features.

The majority of high-throughput sequencing research, in all areas of microbiology, within the past five years has gone into metatranscriptomics, which uses polyadenylated RNA (mRNA) as a sequencing template, to search for key cellulolytic genes within the rumen microbiome (Qi *et al.* 2011). Hess *et al.* (2011) used pyrosequencing to characterize biomass-degrading genes and genomes. They were able to identify 27,755 putative carbohydrate-active genes and expressed 90 candidate proteins. Furthermore they were able to assemble 15 uncultured microbial genomes, which were validated by single-cell genome sequencing. Qi *et al.* (2011) also used metatranscriptomics to investigate the functional diversity of eukaryotic microorganisms within the rumen of muskoxen with a focus on plant cell wall degrading enzymes. From these data, 59,129 contigs were assembled and over 2,500 contigs helped identify a number of plant cell wall degrading enzymes which have rarely been described in previous metagenomic studies. However, our basic understanding of the rumen microbiome is still limited and it is important to first fully understand the content and diversity of rumen microbes before we will be able to manipulate the rumen in a manner to improve nutrition for the host.

The relatively large number of unidentified microbial species, despite the development of molecular techniques for identification, is related to the relative youth of this field of research. Therefore, it is still necessary to continue aggressive sequencing efforts in the rumen to cover the broader range of molecular diversity. However there are limitations to these sequencing efforts which include the need to culture bacteria for sequencing, and the inability of sequencing to infer the bacterial function within the rumen. Therefore it is also necessary to pursue the advancement of sequencing techniques and overcome current sequencing limitations. Projects such as the Hungate 1000, which are attempting to produce a reference set of rumen microbial genome sequences by sequencing the genomes of available cultivated rumen bacteria and methanogenic archaea, together with representative cultures of rumen anaerobic fungi and ciliate protozoa will be necessary for the continued advancement of the science of rumen microbiology.

2.7 Summary

Molecular techniques have revealed greater diversity in the rumen microbial populations than realized with traditional culturing techniques. However, current knowledge of rumen microbial ecology is still limited owing to the extremely complex microbial interactions that occur within this environment. The detection methods for studying rumen microorganisms have moved from limited capability cultivation-based techniques to more sensitive and accurate molecular methods like 16S rRNA, DGGE and TRFLP for determination of population shifts, real-time PCR for identification and enumeration of individual microorganisms with known metabolic properties (Hill *et al.* 2004; Ferrer *et al.* 2005; Shi *et al.* 2007).

Alteration of the rumen bacterial community by dietary manipulation may be less than expected when compared with variability within individual hosts. Such small changes in microbial diversity are likely below the detection limits of conventional molecular techniques,

such as DGGE, TRFLP and clone libraries. Therefore, future studies should be directed toward the use of next generation sequencing techniques to help elucidate unculturable species and species below the limit of detection of other molecular methods. Though very little research has been able to elucidate the exact sensitivity of next generation sequencing, it has been shown to increase sensitivity over other extremely sensitive molecular methods such as microarrays (Mardis *et al.* 2008). It is also important to increase the breadth of coverage with these techniques to help characterize the microbial population dynamics among a greater number of animals, at different times, over a wider range of diets. With more powerful and systematically available molecular techniques, there is increasing availability to integrate microbiological and nutritional objectives in future research. Greater knowledge of the diversity and function of microbial communities should allow more opportunities to account for variation among diets, animals, and experiments for better prediction of rumen digestibility of nutrients and an increased understanding of the events involved in dietary disruption of the ruminal ecosystem. This will help researchers and producers meet the societal demands for improved efficiency of nutrient usage, increased animal welfare and more desirable animal products.

2.8 Hypothesis and Objectives

Rumen microbial populations will be less similar between animals on the same diet than within an animal on different diets. The populations for the solid, liquid and epithelial rumen populations become less diverse as forage is removed from the diet and pH decreases. The inherent microbial population of the rumen will decrease in diversity and total number as the result of an acidotic challenge. The severity of an acidotic challenge on the individual animal will correlate to the presence or absence of specific rumen bacteria.

The objectives of this research are to determine the following:

1. To identify changes in microbial populations in response to diets that potentially predisposes feedlot cattle to acidosis.
2. To determine the inherent variability in rumen microbial populations within and among animals in response to dietary changes in all compartments of the rumen (solid associated, liquid associated and epithelial adherent).

Chapter 3 describes the analysis of the rumen microbial composition using two common molecular techniques (DGGE and real-time PCR). This paper is the introduction to non-culture based rumen microbial analysis in cattle fed different diets. Chapter three meets the objective of identifying changes in the microbial populations in response to diets which may predispose feedlot cattle to acidosis.

CHAPTER 3

3.0 CHARACTERIZATION OF RUMEN BACTERIAL DIVERSITY IN FEEDLOT CATTLE[1]

3.1 Introduction

Ruminal bacteria are vital to the health and productivity of the host (Russell 2002; Welkie *et al.* 2010). Rumen microbiota are known to be highly responsive to changes in diet, age, and health of the host animal (Kocherginskaya *et al.* 2001; Li *et al.* 2009). Therefore, in healthy growing cattle, diet composition is the most important driver of hierarchical structural changes in bacterial populations (Welkie *et al.* 2010). By increasing the availability of fermentable carbohydrate, microbial growth is stimulated. This results in an increase in the rate of fermentation providing the animal with increased energy for growth (Nagaraja and Titgemeyer 2007). However, the proliferation of rumen cellulolytic organisms is directly correlated to the amount of fiber in the diet and replacement of fibre with more readily fermentable carbohydrates impacts these organisms and alters the dynamics of the rumen ecosystem (Tajima *et al.* 2001, Klieve *et al.* 2003). Increased digestible carbohydrate intake has been associated with acidic rumen conditions (pH < 6.0), which have been shown to reduce the activity of fibrolytic bacteria and increase the activity of amylolytic and lactic-acid utilizing bacteria in the rumen (Russell 2002; Klieve *et al.* 2003; Nagaraja and Titgemeyer 2007). As rumen microbial ecologists have been attempting to discover "who is there" (population), in what abundance (richness) and "what they are doing" (community structure) it has been found that the rumen microbial community changes at both the structural and population levels with diet change. However, the specifics of

[1] A version of this chapter has been published. Petri, R.M., Forster, R. J., Yang, W., McKinnon, J. J. and T. A. McAllister. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *J Applied Micro.* Vol 112 (6) pp. 1152-1162

these changes are not well understood due to the extreme complexity of the rumen ecosystem (Li *et al.* 2009).

Culture-dependent studies have shown modest changes in the total culturable bacterial species in the rumen. However, changes in the entire bacterial community, including both culturable and unculturable species are largely uncharacterized (Kobayashi 2006). Detection methods for studying rumen microorganisms have recently moved from the limited capability of cultivation-based techniques to more sensitive molecular methods that allow the determination of community diversity and richness, and can indirectly quantify populations without culturing (McSweeney *et al.* 2007). Understanding how dietary changes impact the rumen ecosystem will provide insight into why certain diets may impact animal health and productivity. The hypothesis of this experiment is that overall bacterial diversity as measured by DGGE and fibrolytic bacteria as measured by real-time PCR will be reduced in animals fed a diet containing no forage as a result of decreased ruminal pH.

3.2Materials and Methods

3.2.1 Experimental Design

This experiment uses a subset of data derived from an experiment by Li *et al.* (2011) which involved eight Angus heifers with ruminal fistulas (initial BW 455 ± 10.8 kg). The heifers were randomly assigned to a replicated 4 × 4 Latin square experiment, balanced for carry over effects. The objective of the original study was to determine if replacing barley grain with wheat DDGS, a non-starch carbohydrate source, influences the animal's susceptibility to sub-acute ruminal acidosis (SARA). The original experiment consisted of four, 21 d periods, each consisting of 12 d of dietary adaptation and 9 d of data collection. The four experimental treatments consisted of barley silage, concentrate (barley grain + supplement), and wheat DDGS in ratios of 15:85:0,

10:65:25, 5:65:30, and 0:65:35 (DM basis), respectively. Comprehensive feed analysis, intake and digestibility, and rumen fermentation data have been published (Li *et al.* 2011).

For the present study, two dietary treatments were selected to study the effects of replacing the forage component and partial replacement of barley grain in a finishing diet with wheat DDGS on bacterial community. These included the 15:85:0 (high concentrate with forage; HC) and the 0:65:35 (high concentrate, no forage; HCNF) diets (Table 3.1). From the original Latin square design, all animals were sampled for this study when fed these two dietary treatments. One heifer was removed from the study due to illness. Animals were fed ad libitum, once daily at 1100 h. The experimental procedures used were approved by the Lethbridge Research Center Animal Care Committee and were in accordance with the guidelines of the Canadian Council of Animal Care (Olfert *et al.* 1993).

3.2.2 Rumen Sampling

Daily feed intake (kg d^{-1}) was calculated as the difference between feed offered and refused during the last 7 d of each period for each individual animal. The composition of experimental diets is given in Table 3.1. Rumen bacterial samples were collected 1 h before and 3 h after feeding on d 14 of each period. Rumen contents were sampled every 2 h over a 24 h period (16 d) for volatile fatty acids (VFA), osmolality and NH_3-N analysis. In-dwelling pH data were collected over 5 consecutive days (12 – 17 d). Ruminal pH was continuously monitored every 30 s for 12 h from d 13 to 18 of each experimental period using the Lethbridge Research Center Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA; Penner *et al.* 2006). The daily ruminal pH data were averaged for each minute and summarized as minimum pH, mean

Table 3.1. Ingredient and chemical composition of the experimental diets[1]

Item	Diets	
	HC	HCNF
Ingredient, % DM		
Barley silage	15	--
Barley grain, temper-rolled	82.8	62.8
Wheat DDGS	--	35
Canola meal	0.50	0.50
Calcium carbonate	1.25	1.25
Molasses	0.12	0.12
Salt	0.15	0.15
LRC beef feedlot premix[2]	0.05	0.05
Urea	0.10	0.10
MGA 100 premix[3] (220 mg/kg)	0.02	0.02
Vitamin E (500,000 IU/kg)	0.003	0.003
Chemical composition[4]		
Dry Matter (DM), %	71.5 ± 0.5	85.1 ± 0.1
Crude Protein, % DM	12.0 ± 0.2	22.9 ± 0.1
Neutral Detergent Fibre, % DM	24.4 ± 0.1	21.8 ± 0.1
Acid Detergent Fibre, % DM	11.1 ± 0.1	10.3 ± 0.2
peNDF[5], % DM	2.81 ± 0.1	0.81 ± 0.1
Ether Extract, % DM	2.3 ± 0.2	3.1 ± 0.2
Starch, % DM	48.9 ± 0.4	34.9 ± 0.4

[1]Ingredient, chemical composition and analysis of feedstuffs have been previously reported as part of a larger study by Li *et al.* 2011.

[2]Supplied per kilogram of dietary DM: 15 mg of Cu, 65 of mg Zn, 28 mg of Mn, 0.7 mg of I, 0.2 mg of Co, 0.3 mg of Se, 6000 IU of vitamin A, 600 IU of vitamin D, and 47 IU of vitamin E.

[3]MGA = Melengestrol acetate.

[4]Values shown with standard error of means.

[5]peNDF was determined by multiplying dietary NDF content by the proportion of the DM retained on the 19- and 8-mm sieves of a Penn State Particle Separator (Lammers *et al.* , 1996).

pH, maximum pH as well as duration and area under the curve below the benchmarks of pH 5.8, 5.5 and 5.2 (Nocek 1997; Penner *et al.* 2007; Beliveau and McKinnon 2009). Li *et al.* (2011) previously reported the collection and analysis of ruminal contents for fermentation measurements. A sub-set of these samples were used in the present study to define the rumen fermentation conditions associated with the microbial population changes reported.

3.2.3 Bacterial Extraction

Particulate and fluid samples from three rumen locations (top, bottom, and middle of the rumen mat) were collected through a cannula, thoroughly mixed and separated into liquid and solid fractions as described by Kong *et al.* (2010). In brief, 100 ml of rumen contents were anaerobically transferred into a heavy-walled 250 ml beaker and squeezed with a Bodum coffee maker plunger (Bodum Inc., Triengen, Switzerland). The liquid fraction was decanted and sub-sampled into 2 ml Eppendorf tubes. The squeezed digesta was washed twice with 100 ml O_2-free phosphate rinse buffer (K_2HPO_4, 30 mmol; KH_2PO_4, 20 mmol; $NaHCO_3$, 35 mmol) by stirring gently with a spatula, followed by squeezing and disposing of the remaining liquid. After washing, 10 ml O_2-free methyl cellulose release buffer containing phosphate rinse buffer with 0.2 % methyl cellulose were blended with residual rumen contents using a Braun hand blender (MR4000, Braun GmbH, Kronberg, Germany) using three 2 s bursts with a 10 s pause in between. The blended digesta were then separated into liquid and particle fractions using a Bodum filter (Kong, 2010). Liquid (5 ml) obtained from the second decanting, containing the particle-associated bacterial fraction were aliquoted into 15 ml falcon tubes. Liquid and solid bacterial fractions were centrifuged at 10,000 × g for 10 min to pellet the bacteria. After the supernatant was discarded, 1.4 ml ASL stool lysis buffer (QIAamp DNA Stool Kit, Qiagen,

Mississauga, ON, Canada) was added to each pellet and the pellet was resuspended. Samples were stored in 2 ml cryogenic tubes at – 80ºC until processed for DNA extraction.

3.2.4 Bacterial DNA Extraction

Rumen samples (n = 64) were thawed at 95°C for 5 min and immediately centrifuged at 10,000 × g for 5 min. Samples were extracted using the method described by Kong *et al.* 2010. In brief, all samples were treated with 0.4 M potassium phosphate buffer, lysozyme (100 mg/ml), mutanolysin (2.5 U μl^{-1}) and proteinase K (20 mg ml^{-1}) prior to bead beating. Glass beads (200 mg with 0.5 mm diameter and 300 mg with 1.0 mm diameter) were added to each tube and the samples were processed in a bead-beating homogenizer (B. Braun, Melsungen AG, Germany) for 3 min and then centrifuged. The supernatant obtained from the pellets after enzyme treatment/bead-beating as well as prior to enzyme treatment and bead-beating were processed using the DNA extraction protocol provided in the QIAamp DNA Stool Mini Kit (Qiagen). After extraction, DNA concentration and purity were assessed using a Synergy HT multi-detection microplate reader (Bio-Tek Instruments Inc., Inc. Winooksi, VT, U.S.A) and gel electrophoresis, respectively. Each sample was divided into two sub-samples for PCR-DGGE and real-time PCR analysis.

3.2.5 PCR-DGGE

Extracted undiluted bacterial DNA (3 µL) from each of the rumen samples was added as template to amplify the V3 region of the 16S rRNA gene for PCR-DGGE reactions in a 25 µl reaction. Amplification was performed using Qiagen HotStar Plus Master Mix Kit (Qiagen) and 500 nM of forward and reverse primers (341f with GC-Clamp: CGCCCGCCGCGCG-

CGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG and 534r:ATTA-CCGCGGCTGCTGG) developed by Muyzer *et al.* 1993. Polymerase chain reaction conditions were 95°C for 5 min, 94°C for 30 s, temperature gradient decreasing from 65°C to 55°C by 0.5°C each cycle for 30 s, 72°C for 1 min for 20 cycles, followed by 94°C for 30 s, 56°C for 30 s, 72°C for 1 min for 10 cycles and 72°C for 10 min for final elongation. The quality of amplified DNA was verified using gel electrophoresis and quantified using flurospectrophotometry by measuring the $A_{260/280}$ (ND-3300 Nanodrop, Wilmington, DE, U.S.A). All amplified DNA was then diluted to a concentration of 400 ng per lane and loaded on 8% acrylamide gels with a 45–60 % denaturing gradient of urea and formamide. Electrophoresis was performed at 60°C and 40V for 20 h. Three lanes on each gel were loaded with DDGE Marker II (Wako, Nippon Gene, Japan) to provide both an internal and external marker. Gels were stained with SybrGold Nucleic Acid Gel Stain (Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A) according to manufacturer's instructions and photographed by UV transillumination.

3.2.6 Real-Time PCR

Quantitative analysis was performed with the ABI PRISM 7700 Sequence Detection System (AB Applied Biosystems, Life Technologies Corp.) to quantify the relative abundance of 16S rRNA genes of seven bacterial species as a percentage of total eubacterial 16S rRNA, using the primers shown in Table 3.2. The quantification of DNA for each bacterial species in rumen contents was performed with Quantifast Kit (Qiagen) using SYBR green chemistry. Standards and samples were assayed in 25 µl reaction mixture containing 15 µl of Quantifast SYBR Green Master Mix, 8 µl of nuclease-free water and 2 µl of DNA template. Amplification programs

Table 3.2. Species and genus specific primers for the quantification of rumen bacteria using real time PCR assay

Target Taxon or Strain	Primer Sequence 5" to 3'	Tm	Amplicon Length	References
General Bacteria	F:GTGSTGCAYGGYTGTCGTCA R:ACGTCRTCCMCACCTTCCTC	61	150	Maeda *et al.* 2003
Ruminococcus Genus	F:GAGTGAAGTAGAGGTAAGCGGAATTC R:GCCGTACTCCCCAGGTGG	60	75	Weimer *et al.* 2008
Selenomonas ruminantium D	F:CAATAAGCATTCCGCCTGGG R:TTCACTCAATGTCAAGCCCTGG	61	82	Stevenson and Weimer 2007; Li *et al.* 2009
Streptococcus bovis	F:CTAATACCGCATAACAGCAT R:AGAAACTTCCTATCTCTAGG	57	869	Tajima *et al.* 2001
Fibrobacter succinogenes S85	F:GCGGGTAGCAAACAGGATTAGA R:CCCCCGGACACCCAGTAT	59	77	Stevenson and Weimer 2007; Li *et al.* 2009
Megasphaera elsdenii	F:AGATGGGGACAACAGCTGGA R:CGAAAGCTCCGAAGAGCCT	59	79	Stevenson and Weimer 2007
Prevotella Genus	F:GGTTCTGAGAGGAAGGTCCCC R:TCCTGCACGCTACTTGGCTG	61	121	Stevenson and Weimer 2007
Ruminobacter amylophilus	F:CAACCAGTCGCATTCAGA R:CACTACTCATGGCAACAT	57	642	Tajima *et al.* 2001

were performed under the following fast conditions: 95°C for 5 min, 95°C for 10 s and a 30 s annealing/elongation (at the temperatures shown in Table 3.2 based on each primer pair) for 40 cycles. The melting curve of PCR products was monitored by slow heating with an increment of 0.1°C s^{-1} from 60 – 95°C with fluorescence collection at 0.1°C intervals to confirm specificity of amplification. A standard curve for each bacterial species was constructed by using plasmid DNA containing 16S rRNA inserts of DNA purified from a pure culture of the target species (Stevenson and Weimer 2007). *Ruminococcus* plasmid DNA was used as a standard template for universal bacteria primers. Plasmid DNA was quantified and then subjected to seven sequential ten-fold dilutions, each analyzed in duplicate. A linear relationship was observed between the threshold cycle (C_t) and log of DNA concentration when each primer pair was tested against purified DNA from its target taxon (r^2=0.97 to 0.99). Each sample was run in triplicate and the PCR reaction cycle at which the reaction exceeded this was identified as the C_t. The copy numbers of total bacteria and each enumerated species, in 20 ng DNA, were determined by relating the C_t values to standard curves based on the following calculation:

DNA (number of molecules) = (6.02×10^{23} (molecules/mol) x DNA amount (ng))/ (DNA plasmid-insert length (bp) x 6.6×10^{11}(ng mol^{-1} bp^{-1}))……..(3.1)

Amplification products were verified by horizontal gel electrophoresis of a 5 μl aliquot in a 1% agarose gel in Tris-Acetate-EDTA (40mM Tris acetate, 1mM EDTA; pH 8.5), followed by ethidium bromide staining and visualisation under UV light. A 1 kb Ladder (Quickload, New England Biolabs Ltd., Pickering, ON, Canada.) was included on each gel to enable confirmation of the size of the amplified product.

3.2.7 Relative Quantification

To minimize errors of absolute quantification of DNA from rumen samples, relative quantification methods were used. In relative quantification, amplification is expressed relative to the amplification of reference primers utilizing experimentally derived amplification efficiency (Pfaffl 2001; Stevenson and Weimer 2007). The proportion of each species was obtained by copy numbers of 16S rRNA gene of targeted species divided by the 16S rRNA genes amplified with a reference primer set (Khafipour *et al.* 2009; Li *et al.* 2009). A non-degenerate, domain-level primer set that amplified all eubacterial species was used as the reference primer set (Table 3.2).

3.2.8 Statistical Analysis

Experiment was analyzed with a fixed effect of treatment (diet) and random effects of animal and time. Data for rumen environmental parameters were analyzed for effect of treatment, time, and treatment x time interaction over the 12 h sampling period using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 5.1, Applied Maths, Inc., Austin, TX, U.S.A) and similarity matrices to identify community population differences between treatments, digesta fractions, and individual animals. Using average Dice's similarity coefficient (D_{sc}) index, with an optimization of 1% and with a tolerance of 1.5%, clustering was carried out using the unweighted pair group method with arithmetic means (UPGMA). Diversity indices were calculated applying the following equations using the band area as determined by BioNumerics software:

Relative Band Area = band area/ Σ(all measured band areas in the sample)…….(3.2)

Shannon-Weiner (H′) = Σ (-(Relative Band Area) (Log_{10}(Relative Band Area)))…….(3.3)

Simpson's Index (λ) = 1- (Σ Relative Band Area in a Sample)^2……(3.4)

Diversity index values were calculated for each sample and analyzed using the one-way ANOVA procedure of SAS. Relative quantities of 16S rRNA as determined from real time PCR were analyzed using PROC ANOM to determine significant differences in copy number between animals, treatments and pH profiles for each targeted bacterial species. Significance level α = 0.05, trends were declared for $0.10 \geq \alpha > 0.05$.

3.3 Results

3.3.1 Rumen Fermentation Characteristics

Cattle gained an average of 123 ± 24 kg over the course of this study. Dry matter intake was not altered by diet (Table 3.3). Duration (min/d) below pH 5.2 was found to be significantly longer (P=0.05) for animals fed a HCNF diet. The duration of time and area under the curve (pH × min) for pH<5.5 also tended to be longer for HCNF (P=0.07 and P=0.06 respectively). Area of the curve below pH 5.2 showed a similar trend (P=0.08) with those animals fed the HCNF diet having a greater total area (28.24 pH × min) compared to those animals fed HC (5.55 pH × min). Lowest mean pH reached each day as indicated by mean nadir was 5.22 and 5.13 for HC and HCNF, respectively. Dietary treatment did not alter ($P > 0.10$) VFA or NH_3-N concentrations (Table 3.3). Conversely, rumen osmolality was significantly higher (P=0.05*)* in those animals fed HCNF as compared to HC.

Table 3.3. Changes in dry matter intake, rumen pH, rumen volatile fatty acids, NH_3-N and osmolality in cattle fed a high concentrate diet and a high concentrate-no forage diet.

	Dietary Treatment			
Fermentation Parameters	**HC[1]**	**HCNF[2]**	**SEM**	***P*-value Treatment**
DMI[3] (kg day^{-1})	9.50	8.77	0.39	0.21
Mean Nadir	5.22	5.13	0.06	0.38
Mean Daily pH	5.94	5.73	0.09	0.11
Rumen pH≤5.8				
Duration (min day^{-1})	580.80	802.80	120.00	0.22
Area Under (pH × min)	180.35	327.05	62.03	0.12
Rumen pH≤5.5				
Duration (min day^{-1})	286.80	541.80	91.80	0.07
Area Under (pH × min)	53.45	142.43	30.86	0.06
Rumen pH≤5.2				
Duration (min day^{-1})	65.40	244.20	57.60	0.05
Area Under (pH × min)	5.55	28.24	8.48	0.08
VFA				
Total, m*M*	122.35	119.18	5.47	0.69
Acetate (A), %	62.67	55.13	4.13	0.22
Propionate (P), %	37.13	39.00	3.66	0.72
Butyrate, %	18.43	22.09	2.00	0.22
A:P (Acetate: Propionate)	1.80	1.50	0.20	0.39
NH_3-N, m*M*	9.09	14.16	2.50	0.18
Osmolality, mOsm kg^{-1}	320.1	340.7	6.80	0.05

[1]HC = high concentrate
[2]HCNF = high concentrate no forage
[3]DMI = dry matter intake

3.3.2 PCR-DGGE

Detectable bacterial PCR-DGGE profiles clustered similarly between the liquid and solid fractions of rumen contents and between the two dietary treatments (Figure 3.1). The average D_{sc} of detectable bacterial profiles among digesta samples collected from the liquid and solid fractions in the rumen ranged from 77.7 to 97.4 % (Table 3.4). Average D_{sc} among samples collected from the HC and HCNF diet ranged from 84.5 to 98.1 % (Table 3.4). Simpson's index showed that heifers fed the HCNF diet exhibited greater (P=0.05) diversity of predominant species (Table 3.5). The Shannon-Weiner index similarly showed that heifers fed the HCNF had a trend (P= 0.06) towards a greater number of unique species (HC =1.26; HCNF=1.35).

3.3.3 Real-Time PCR

The real-time PCR results are summarized in Figure 3.2. Seven bacterial species evaluated in this study were detected in all heifers, in both diets and in both the solid and the liquid fractions of rumen contents. Quantities of bacteria expressed as a percentage of total enumerated bacteria ranged from 0.0001 to 70.2 % between diets and from 0.0006 to 63.6% between the solid and the liquid fractions of rumen digesta (Table 3.6). There was a 57-fold decrease in relative abundance of the *Fibrobacter succinogenes* populations (P=0.01) in cattle fed the HCNF diet (Table 3.6). When treatments were compared, only *F. succinogenes* accounted for a higher (P=0.01) proportion of the total bacteria (1.14 %) in the HC diet as compared to the HCNF diet (0.02 %). *Megasphaera elsdenii* tended to be higher (P=0.09) in the HCNF diet (6.5 fold increase), whereas *Ruminobacter amylophilus* tended to be higher (P=0.08) in cattle fed the HC diet (12 fold increase; Table 3.6).

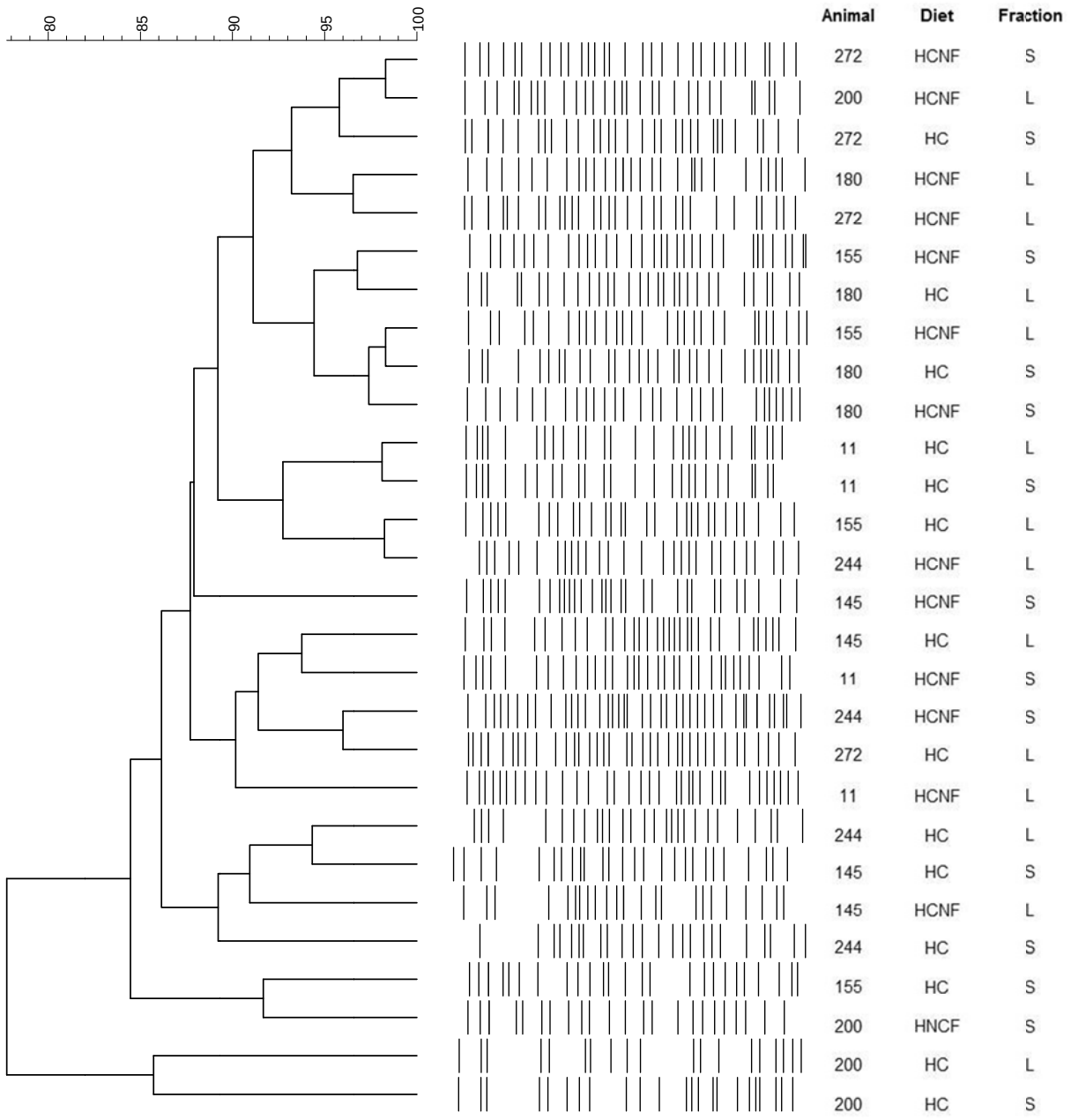

Figure 3.1. Cluster analysis of DGGE-PCR fingerprint profiles similarity (%) for all heifers, fed both HC and HCNF diets, for both the solid (S) and liquid (L) fractions of rumen digesta. Clustering was done using Dice's algorithm and UPGMA at an optimization of 1 %, a tolerance of 1.5 % and clusters were considered similar at 90 % or higher.

Table 3.4. Average D_{sc} (%) in detectable bacterial diversity profiles (DGGE) among rumen digesta samples, across treatments based on the liquid versus solid fractions of digesta and diet. Similarity was measured at 90 %.

	Digesta Fraction		Dietary Treatment	
Animal ID	**Solid**	**Liquid**	**HC**[1]	**HCNF**[2]
11	87.7	87.7	98.1	90.2
145	86.2	86.2	86.2	86.2
155	84.5	89.2	84.5	94.4
180	97.4	91.1	94.4	91.1
200	77.7	77.7	85.7	77.7
244	86.2	86.2	89.2	87.7
272	95.8	87.7	87.7	93.2

[1]HC = high concentrate
[2]HCNF = high concentrate no forage

Table 3.5. Diversity and Dominance Indices calculated from DGGE incidence profiles of rumen fluid from the two diets

	Dietary Treatment			
Diversity Measure	**HC**[1]	**HCNF**[2]	**SEM**	***P*-value**
Number of Bands	27.4	29.5	1.11	0.17
Shannon-Weiner Index	1.26	1.35	0.032	0.06
Simpson's Index	0.93[a]	0.94[b]	0.006	0.05

[1]HC = high concentrate
[2]HCNF = high concentrate no forage

Table 3.6. Effect of diet and digesta fraction on the percent of total enumerated eubacteria 16S rRNA genes of dominant rumen bacterial species using quantitative real-time PCR.

	Dietary Treatment[1]				Digesta Fraction[1]			
Bacteria	**HC[2]**	**HCNF[3]**	**SEM**	***P*-value**	**Liquid**	**Solid**	**SEM**	***P*-value**
Ruminococcus spp.	0.71	0.71	0.153	0.97	0.40a	1.01b	0.196	0.004
Fibrobacter succinogenes	1.14a	0.02b	0.219	0.01	0.20a	0.96b	0.219	0.02
Prevotella spp.	56.57	70.22	6.924	0.18	63.23	63.56	6.924	0.97
Selenomonas ruminantium	2.41	1.69	0.534	0.26	0.87a	1.73b	7.682	0.05
Megasphaera elsdenii	0.12	0.78	0.286	0.09	0.24	0.65	0.286	0.28
Ruminobacter amylophilus	0.0012a	0.0001b	>0.0001	0.08	0.08	2.34	1.404	0.86
Streptococcus bovis	0.5	1.98	1.404	0.46	0.0007	0.0006	0.0004	0.89

[1]No significant diet by digesta fraction interaction
[2]HC = high concentrate
[3]HCNF = high concentrate no forage

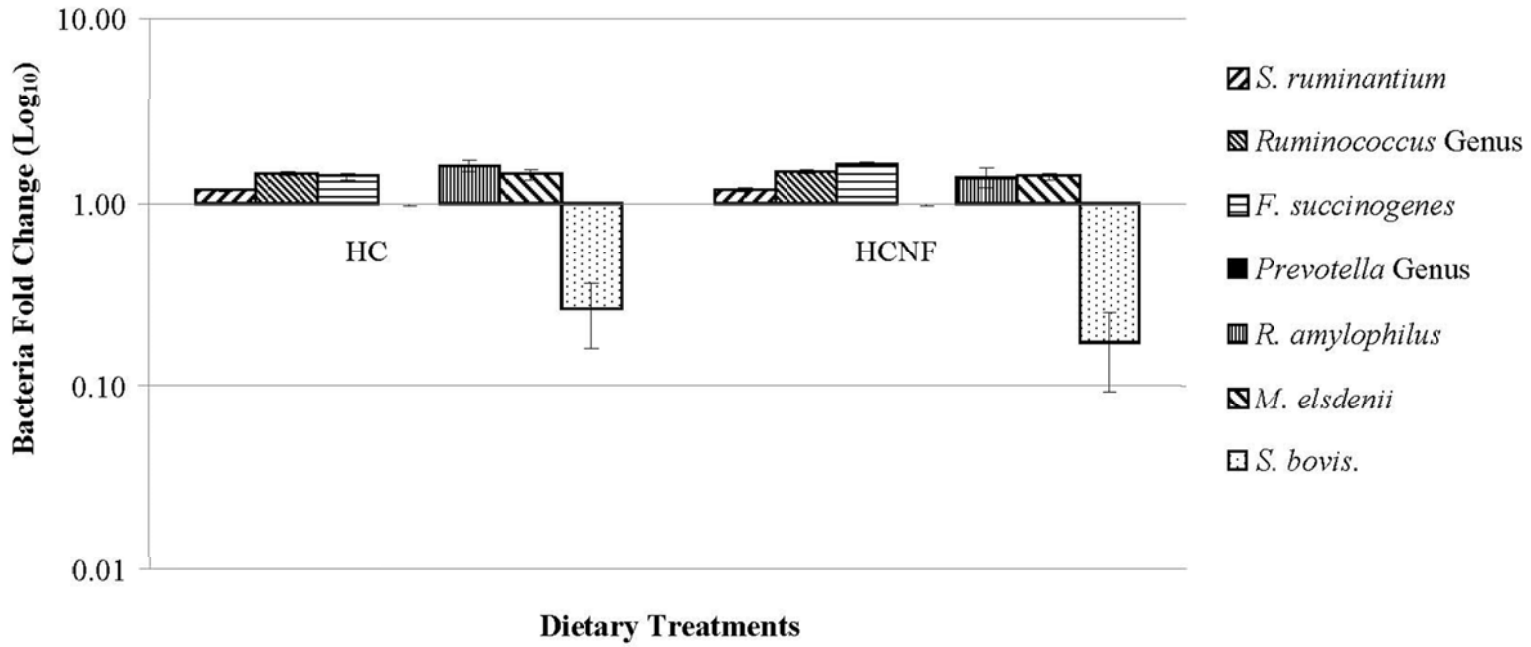

Figure 3.2. Fold changes in relative quantification of populations of enumerated bacteria species between diets. Basal frequency represents total bacterial enumerated using the non-degenerate domain-level reference primer set.

Ruminococcus spp. (P<0.01) and *Selenomonas ruminantium* (P=0.05) were both more than 1.4 fold higher in the solid fraction compared with the liquid fraction of rumen contents (Table 3.6). The amount of *F. succinogenes* associated with the solid fraction was significantly higher (P<0.05) than with the liquid fraction. No significant treatment by digesta fraction interaction was found.

3.4 Discussion

Using current molecular techniques, it was possible to study the impact of the removal of the forage component of the diet on the diversity and stability of rumen bacterial populations. Composition of the diet, particle size and intake are critical factors in determining the impact of diet on rumen microbial activity, nutrient digestion and rumen function (Nagaraja and Titgemeyer 2007). The diets used in this study met or exceeded the energy and protein needs for maintenance and growth of the animals used in this study (NRC 2000). Barley grain and wheat DDGS have been shown to have similar digestible energy values in ruminant diets (Beliveau and McKinnon 2008). Therefore, as a result of the replacement of silage with DDGS, the HCNF diet would be slightly higher in metabolizable energy content than the HC diet. The two diets also differed in the form of energy supplied (i.e. starch vs. fermentable fibre, protein and fat).

3.4.1 Rumen Fermentation Characteristics

Reducing the starch content of the diet by substituting wheat DDGS for grain and silage did not influence dry matter intake. A similar response has been observed in other studies where wheat DDGS replaced barley grain at similar levels (Beliveau and McKinnon 2008; Gibb *et al.* 2008). There was an indication that substitution of DDGS for part of the barley and all of the forage increased the duration that ruminal microorganisms were exposed to low pH. This

substitution reduced the mean particle size in the diet, increasing the total surface area available for microbial colonization and reducing rumination (Allen 1988). Reduced rumination would also be a consequence of the 3.5 fold decrease in physically effective fiber (peNDF) in the HCNF diet (Table 3.1). Reduced peNDF would also result in lower saliva secretion and associated buffering within the rumen. Under this scenario, ruminal pH can decline (Ørskov 1999) even if VFA concentrations are similar between diets as was the case in our study. Furthermore, while DDGS is high in neutral detergent fibre (NDF) it is low in lignin and high in digestible fibre (i.e. 62 to >71 %) (Birkelo *et al.* 2004; Vander Pol *et al.* 2009; Klopfenstein *et al.* 2008). The highly digestible nature of the HCNF diet could also have increased the duration that microbial populations were exposed to low pH. The negative effect of feeding wheat DDGS on rumen pH, particularly on the duration of pH under 5.2 and area under the curve for pH 5.2 has been reported when DDGS has been used to replace barley grain (Beliveau and McKinnon 2009) and barley silage (Wierenga *et al.* 2010).

3.4.1 PCR-DGGE

Detectable bacterial PCR-DGGE profiles between treatments were statistically similar however some liquid and solid fractions from the same diet clustered more closely, whereas others showed no similarity (Figure 3.1). Failure to see dietary differences in clustering may be a result of the fact that both diets were high in concentrate and low in effective fibre. As a result, individual animal variation may have masked any diet effects on bacterial populations. This would explain why our results differ from Kocherginskaya *et al.* (2001) who showed a significant clustering effect between forage (100 % hay) and concentrate (72 % grain) fed cattle. Supporting this conclusion, Li *et al.* (2009) using PCR-DGGE methods showed high individual animal variation as well as animal specific clustering in cattle fed a ration of 55 % concentrate

and 45 % forage. Animal variation within this experiment was reflected not only in DGGE-PCR similarity coefficients (Table 3.4), but also in the high variation in pH measurements. The high variation in pH reflects the different abilities of individual animals to cope with dietary change and imbalances in the production and absorption of VFA's (Brown *et al.* 2000, Bevans *et al.* 2005; Penner *et al.* 2009). Thus, even when treatment means were similar, irregular variance from the mean reflects the differential extent to which animals are able to compensate or tolerate the change in rumen fermentation conditions associated with a change in dietary substrate (Bevans *et al.* 2005).

According to DGGE, bacterial composition of the rumen is represented by a limited number of OTU's as the total band number indicating individual OTU's, did not differ significantly between the two diets though heifers fed HCNF numerically had more bands than the HC diet (Table 3.5). Multiple methods of diversity analysis were calculated to increase the confidence level of the conclusions. Shannon-Weiner diversity index and Simpson's index both showed a trend towards greater microbial diversity and a greater diversity in predominant species in heifers fed the HCNF diet. These results contradict our hypothesis that the removal of forage would reduce microbial diversity. However, it should also be noted that removal of the silage did not completely remove dietary fiber. The HCNF diet still had a significant structural carbohydrate fraction although derived from a different source (i.e., wheat DDGS) and a considerably higher protein content. As a result fibrolytic bacteria may still have remained active against fermentable fiber and an increase in other bacteria due to increased fermentable substrates may have contributed to the increased diversity observed. Additionally, it can be hypothesized that the removal of forage and trend towards a decrease in DMI may have impacted ruminal rate of passage and therefore provided longer growing times for metabolically slower bacteria. While

this methodology provides a rapid and repeatable characterization of the system, it is important to note that the level of resolution offered by DGGE in its application to the ruminal microbiota analysis remains low. This is in part due to the co-migration of DNA along the gel resulting in bands that may contain multiple bacterial species. Furthermore, the use of touchdown PCR in DGGE selectively amplifies the most abundant phylotypes and therefore resolution of diversity is decreased (Kocherginskaya, 2001).

3.4.2 Real-Time PCR

The prevalence of *F. succinogenes* according to relative quantification was decreased 57-fold in animals fed the HCNF diet. Similar results were reported in other studies where cattle were switched from a forage- to a high concentrate-based diet (Tajima *et al.* 2001; Fernando *et al.* 2010). *Fibrobacter succinogenes* is one of the most active cellulolytic bacteria and is known to adhere to the most fibrous components of the diet (Halliwell and Bryant, 1963; Koike and Kobayashi 2009). It is therefore not surprising to see the drop in numbers of this species with the diet containing no forage. Supporting this conclusion is the fact that *F. succinogenes* was 4.8-fold higher in the solid versus the liquid fraction of digesta. However, *F. succinogenes* and other cellulolytic species such as *Ruminococcus* spp. did not completely disappear from the diets containing no forage. Therefore, the significant decrease in *F. succinogenes* may be a reflection of the highly digestible nature of fiber in DDGS more than the removal of silage as a substrate. *Ruminobacter amylophilus* also showed a trend towards decreased levels in the HCNF diet. *Ruminococcus amylophilus* is an obligate anaerobe that uses only α-linked glucose molecules like maltose, maltodextrins, and starch as a source of energy (Anderson 1995). Therefore, the trend towards lower levels of *R. amylophilus* with the replacement of barley, a high starch feed grain by low starch DDGS is expected. No other real-time PCR primers sets were able to detect a

diet effect (*Ruminococcus. S. ruminantium, M. elsdenii, S. bovis,* and *Prevotella*; Table 3.6). For *Ruminococcus* spp. the similarities between diets may be due to amylolytic capabilities. However, the complete lack of a diet effect was unexpected because like *F. succinogenes* they are known to be primarily fibrolytic species (Koike and Kobayashi 2001). However, similar to *F. succinogenes, Ruminococcus* spp. were noted to be 2-fold higher in the solid than the liquid fraction of the digesta, likely due to their pivotal role in the initial establishment of rumen biofilms on cellulosic feedstuffs (McAllister *et al.* 1994). The lack of dietary effect may be due to the fact that genus level primers were unable to detect important species level changes in *Ruminococcus*. Similar to *Ruminococcus* spp., there was a (1.9-fold) higher count of *S. ruminantium* in the solid fraction of digesta compared with the liquid fraction. This may be explained by the secondary fermentative action of *S. ruminantium* and their limited survival capabilities in the absence of nutrients provided by primary colonizing populations of fibrolytic bacteria (McAllister *et al.* 1994). *Selenomonas ruminantium* has been found to synthesize propionate, malate, and lactate from products of primary fermentation such as pyruvate and succinate (Hungate 1966; Evans and Martin 1997). Unlike *Megasphaera elsdenii,* which shows no catabolite repression by carbohydrates such as glucose and maltose, *S. ruminantium* first ferments glucose, sucrose, and xylose before fermenting DL-lactate which due to competition from other species may have also resulted in a decrease in its level in the liquid fraction (Counotte 1981).

No effect of treatment or distribution between liquid and solid fraction of rumen contents was found for *Prevotella*. However, *Prevotella* was the most dominant group within the rumen bacterial community, accounting for as much as 56% of the total bacteria DNA enumerated in the HC diet and up to 70 % in the HCNF. Consistent with our results, Stevenson and Weimer

(2007) reported *Prevotella* spp. accounted for up to 60% of the total enumerated bacteria when using primers specific to the genus level. *Streptococcus bovis* and *M. elsdenii* were also not affected by treatment or digesta sample fraction. *Streptococcus bovis* counts showed a large standard error, likely due to the primer used to amplify its 16S rRNA (Tajima *et al.* 2001). This primer was designed to amplify a fragment of approximately 800 bp which is more than 4 times larger than recommended for efficient real-time PCR quantification. When results were adjusted for PCR efficiency, the standard error was amplified. However, it is commonly reported that there is no significant difference in *S. bovis* in animals regardless of diet unless the animals are clinically acidotic (Goad *et al.* 1998; Klieve *et al.* 2003; Al Jassim *et al.* 2003; Fernando *et al.* 2010). Differences in dietary protein content between the HC and HCNF treatments were hypothesized to impact the proteolytic populations of the rumen. From the real-time PCR analysis the major proteolytic bacteria quantified were *Prevotella* spp., *Streptococcus bovis* and *Selenomonas ruminantium* (Russell 1981), but these genera did not exhibit major differences across diets. Due to the quantification of only a limited number of rumen species using real-time PCR analysis and limitations in the specificity of DGGE analysis, the differences in microbial diversity between the HC and the HCNF diets seen with the Shannon-Wiener and the Simpson's indices could not be directly correlated.

3.5 Conclusions

Using, DGGE the current study showed that bacterial diversity was not reduced when DDGS replaced a portion of the barley and all of the silage in a finishing diet, despite increasing the duration that the microbial community was exposed to a low pH. In fact, both ecological diversity indices showed an increased number of unique species in diets containing no forage. Quantitative real-time PCR analysis clearly showed that key cellulolytic species decreased in

relative quantities when forage was removed from the diet. However, due to a limited number of bacteria enumerated using real-time PCR it was not possible to document if this led to a greater diversity using real-time PCR.

Chapter 4 describes the analysis of the rumen epithelial microbial composition using the two previously used molecular techniques (DGGE and real-time PCR), as well as the newer technique of pyrosequencing. This paper meets the objective of identifying changes in the microbial populations in response to diets which may predispose feedlot cattle to acidosis as well as determining the inherent variability within the epithelial populations.

CHAPTER 4
4.0 CHANGES IN THE RUMEN EPIMURAL BACTERIAL DIVERSITY OF BEEF CATTLE AS AFFECTED BY DIET AND INDUCED RUMINAL ACIDOSIS[2]

4.1 Introduction

The rumen epithelial, or epimural, bacterial community is a critical component of proper rumen function and performs a variety of functions necessary for host health including the hydrolysis of urea, scavenging of oxygen and the recycling of epithelial tissue (McCowan *et al.* 1978; Cheng and Wallace 1979; Dinsdale *et al.* 1980). Early studies, using electron microscopy and culture-dependent methods clearly showed that bacterial populations adherent to the rumen wall were distinct from those associated with rumen contents (Cheng and Wallace 1979; McCowan *et al.* 1980). Rumen epimural communities were found to be predominantly comprised of Gram-positive species including *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Lactobacillus*, and *Propionibacterium* (Cheng and Wallace 1979). However, these culture-based techniques likely underestimated the biodiversity of the epimural biofilm because it can be difficult to discriminate between species that are closely related, and many members of this community are likely unculturable in the laboratory. As a result numerous members of the rumen epimural community remain uncharcterized (Kobayashi 2006).

More recent publications based on cloning and sequencing of the 16S rRNA gene corroborate that the rumen epithelial bacterial populations differ from those associated with rumen contents (Cheng *et al.* 1980; Chen *et al.* 2011). Molecular techniques have clearly shown that the epimural bacterial community is far more diverse than originally surmised on the basis of electron microscopy (Chen *et al.* 2011). However, variables that may influence the species

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composition of epithelial biofilms such as pH, aerotolerance, nutrient absorption, epithelial cell turnover, digesta passage, and host-communication remain largely undefined. While diet is a major factor influencing the populations and metabolic function of rumen content-associated microbial populations (Kocherginskaya 2001; Tajima *et al.* 2001), it is not known if it has a similar effect on the composition of the epimural bacterial community (Sadet-Bourgeteau *et al.* 2010).

One of the most studied members of the epimural community is *Fusobacterium necrophorum*, a Gram-negative, aerotolerant anaerobe that is present in both digesta and epithelial bacterial communities (Nagaraja *et al.* 2005; Tadepalli *et al.* 2009). This bacterium can become an opportunistic pathogen as it is the primary causative agent of liver abscesses in cattle fed high grain diets (Tadepalli *et al.* 2009). Numbers of *F. necrophorum* are believed to increase as a result of inclusion of grain in the diet (Tan *et al.* 1994). However, as rumen pH decreases during acidosis, *F. necrophorum* becomes undetectable in rumen contents, presumably because of its sensitivity to low pH (Tadepalli *et al.* 2009). Because ruminal acidosis is a predisposing factor for liver abscess it has been hypothesized that *F. ncecrophorum* may persist in the rumen through association with the rumen wall where pH may be moderated as a result of bicarbonate exchange at the apical surface of the epithelial cells (Narayanan *et al.* 1997).

The objective of this research was to characterize the composition of the adherent epithelial bacterial community during dietary adaptation from forage- to a grain-based diet. Changes in epithelial bacterial communities were further monitored during and after recovery from an episode of ruminal acidosis induced using a challenge model, with the objective of defining epithelial bacterial populations that are indicators of acidosis.

4.2 Materials and Methods

4.2.1 Animals and Sampling

This study involved a subset of data derived from an experiment using 16 ruminally cannulated Angus heifers (Schwaiger *et al.* unpublished). All heifers were cared for in accordance with the guidelines of the Canadian Council on Animal Care (Olfert *et al.* 1993). The present study used only eight of these heifers, assigned to one of four blocks based on body weight (BW: 308kg ± 35 SD) to ensure all blocks were weighted similarly. Each block received a progression of 5 dietary treatments over an 11 wk period. Rumen epithelial biopsies, VFA and lactic acid samples were collected from each heifer during each dietary treatment via the rumen canulae. Heifers were fed grass-hay (forage) with a mineral supplement and had no access to grain prior to this experiment. Heifers were fed the grass-hay diet with supplement (Table 4.1) for a minimum of 3 wk before being sampled (d -1). They were then transitioned using a single step to a mixed forage – concentrate (mixed forage) diet consisting of 60% barley silage, 30% barley grain and 10% supplement (dry matter basis). Heifers received the mixed forage diet for 2 weeks prior to the second sampling and were subsequently transitioned over 20 days to a high grain diet (high grain) consisting of 81% barley grain, 9% barley silage and 10% supplement (dry matter basis). After transition, they were fed this diet for a period of 34 d to allow the rumen microbiome to stabilize before the third

Table 4.1. Ingredient composition of diets.

	Dry matter basis (%)		
Ingredient	Forage	Mixed forage	High grain
Grass hay	95.0	--	--
Barley silage	--	60.0	9.0
Barley grain	--	30.0	81.0
Supplement[1]	5.0	10.0	10.0

[1] Supplement composition (% DM): canola meal (33.0), beet pulp (50.0), calcium carbonate (12.0), salt (1.6), Lethbridge Research Centre premix (0.5), urea (2.5), Melengestrol acetate 100 (200 mg/kg; Pfizer Canada Inc., Kirkland QC) (0.3), dry molasses (0.1).

sample was collected (d 69). One week later the heifers were subjected to an acidotic challenge (acidotic challenge) on d 76. The challenge model consisted of restricting each individual heifers feed intake on the challenge day, 1 h prior to the regular allotment of feed for ad libitum intake. The intake restriction, the day before the challenge, was set to 50% of the average ad libitum as-fed intake, based on a percentage of body weight for each individual heifer. Average intake was determined for each heifer using the individual feed intake (as-fed) 31 days prior to the challenge. Body weight was recorded on the first day of the high grain period, 4 d before the challenge and weekly thereafter. After 24 h of feed restriction, a single dose of ground dry-rolled barley grain ground through a 4.5 mm screen was introduced directly through the rumen cannula. In an effort to simulate acidosis, heifers in replicate 1 received a dose of 20% I: BW (as-fed). However, as this challenge was found to be too severe based on low ruminal pH, the remaining heifers received a dose of 10% I: BW (as-fed). Beginning at the time of challenge, then every 2 h for the first 12 h and then every 4 h for the next 12 h, rumen pH was monitored using strained ruminal fluid from the ventral sac and a portable pH meter (Accumet 25, Fisher Scientific). If ruminal pH was below 4.2, an additional pH measurement was made 1 h later. If ruminal pH remained $\leq$ 4.2, heifers were dosed with 250 g of sodium bicarbonate in accordance with animal care guidelines. Heifers were then offered a quantity of feed equivalent to their previous ad libitum intake (based on intake the week prior to the challenge) 1 h after the challenge. Rumen epithelial samples were also collected 1 wk post-challenge (challenge recovery) to gage the degree of recovery after the challenge while the heifers continued to receive the high-grain diet. Composition of the diets is in Table 4.1.

4.2.2 Rumen Sampling

Rumen contents were sampled at the same time as bacterial sampling (4 h post feeding) for measurement of volatile fatty acid (VFA) and lactic acid concentrations. In-dwelling ruminal pH was recorded on the day of sample collection (every minute) starting at 0800 h. A more extensive study of the diurnal pattern of VFA concentrations and ruminal pH is presented by Schwaiger *et al.* (unpublished). The pH measurements were done using the Lethbridge Research Center Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA) (Penner *et al.* 2006). The daily ruminal pH data were summarized as minimum pH, mean pH, maximum pH as well as duration and area under the curve below the benchmarks of pH 5.8, 5.5 and 5.2 (Penner *et al.* 2006).

4.2.3 Epithelial Biopsies

Ruminal papillae biopsies were collected from the ventral sac of the rumen, after partial content evacuation 4h post-feeding. The ventral sac was landmarked at approx. 25 cm below the bottom of the cannula, and then manually externalized. A 4 × 4 cm area around and including the site of biopsy was rinsed using a syringe with 10 mL of sterile physiological saline to remove attached feed particles, loosely adherent bacteria and any residual rumen fluid. The biopsy was then taken by removing a small portion (1 cm^2) of the epithelium, taken as close to the rumen wall as possible without removing the base of the papillae. Dissection was performed using Kelly forceps and curved dissection scissors (Fisher Scientific Ltd, Nepean, Ontario, Canada). Ruminal papillae were excised, weighed, and flash-frozen in liquid nitrogen and stored at -80°C until DNA extraction.

4.2.4 Bacterial DNA Extraction and Pyrosequencing

Genomic DNA was extracted as described by Kong *et al.* (2010). Briefly, each sample of rumen epithelial tissue (~200 mg) was manually ground to a fine powder in liquid nitrogen using a mortar and pestle, combined with proteinase K (1 mg/mL; Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) and further ground in liquid nitrogen using a Retsch RM100 grinder (Retsch GmbH, Haan, Germany). Samples were processed individually with sterilization of the grinder (12% aqueous sodium hypochlorite; followed by exposure to 15 min of UV radiation) between each sample. Samples were mixed with ~100 mL of liquid nitrogen and then transferred to a 200 mL wide-mouth centrifuge bottle and incubated for 40 min in a 50°C water bath to thaw the samples. After incubation, 15 mL of sample was transferred into a 50 mL polycarbonate tube (SS34; Fischer Scientific Ltd, Nepean, Ontario, Canada) containing 1.5 mL of 20% vol/vol SDS (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). The resultant mixture was then incubated for 45 min at 65°C in a water bath. After incubation, samples were centrifuged at 10,000 × g for 10 min and three equal volumes of supernatant were combined with a preheated (65°C) 2% agarose mixture (Sigma-Aldrich Canada Ltd., Oakville, Ontario). The suspension was gently inverted to create a homogenous mixture and transferred to petri dishes (15 mm H; Fischer Scientific Ltd, Nepean, Ontario, Canada) and allowed to cool at room temperature. Once set (1 h) agarose samples were cut into strips (1 cm wide) and washed in 10 volumes of TE buffer (10:2 of 1M Tris-HCl to 0.5M EDTA) for 16 h. Agar (200 mg) containing cleaned sample DNA were distributed between triplicate 1.5 mL snap cap tubes (Fischer Scientific Ltd, Nepean, Ontario, Canada) and placed in -80°C for 1 h. Frozen samples were "freeze-squeezed" (Thuring *et al.* 1975) by centrifuging at 10,000 × g for 10 min to extract the DNA fragments from the agar. The resulting supernatant of TE buffer containing bacterial DNA was transferred to a new 1.5 mL tube. Samples were then refrozen at -80°C for 1 h and centrifuged once again. Supernatants

were combined from the repeat centrifugation and all samples were stored at 4°C prior to analysis. DNA from each sample was quantified using fluorometric dsDNA using picogreen dye (Invitrogen, Life Technologies Inc., Burlington, ON, Canada) and measured with a synergy HT plate reader (BioTek U.S. Ltd, Winooski, VT, United States). Subsequently, individual genomic DNA samples for all treatments were diluted to a concentration of 20 ng μL^{-1} in TE buffer. One 20 µL aliquot of each sample for a total of 36 genomic DNA samples (forage n=5; mixed n=8; high grain n=7; acidotic challenge n=8; challenge recovery n=8) were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for pyrosequencing using a 454 GS FLX Titanium Sequencing System (454 Life Sciences, a Roche company, Branford, CT, USA). Pyrosequencing targeted the V1 to V3 hypervariable region of the 16S rRNA gene as described by Dowd *et al.* (2008).

4.2.5 PCR-DGGE Analysis

Extracted, diluted DNA (3 µL of 20 ng μL^{-1}) from each sample was added as template to amplify the V3 region of the 16S rRNA gene for PCR-DGGE analysis in a 25 µl reaction. Amplification was performed using Qiagen HotStar Plus Master Mix Kit (Qiagen) and 500 nM of forward and reverse primers (341f with GC-Clamp:CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGG CAGCAG and 534r:ATTACCGCGGCTGCTGG) as previously reported (Kocherginskaya, 2001). Polymerase chain reaction conditions were 95°C for 5 min, 94°C for 30 s, temperature gradient decreasing from 65°C to 55°C by 0.5°C each cycle for 30 s, 72°C for 1 min for 20 cycles, followed by 94°C for 30 s, 56°C for 30 s, 72°C for 1 min for 10 cycles and 72°C for 10 min for final elongation. Amplified DNA was assessed for quality using gel electrophoresis and

quantified using flurospectrophotometry by measuring the $A_{260/280}$ (ND-3300 Nanodrop, Wilmington, DE, U.S.A). Amplified DNA was then normalized to 100 ng μL^{-1} and 4 µL DNA along with 4 µL of 2× loaded dye (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol w/v in H_2O, pH 8.0) were put into each lane on 8% acrylamide gels with a 45 – 60% denaturing gradient of urea and formamide. Electrophoresis was performed at 60°C and 40V for 20 h. Three lanes on each gel were loaded with DGGE Marker II (Wako, Nippon Gene, Japan) to provide both an internal and external marker. Gels were stained with SybrGold Nucleic Acid Gel Stain (Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A) according to manufacturer's instructions and photographed by UV transillumination.

4.2.6 Real-time PCR

Quantitative analysis of the relative abundance of 16S rRNA genes of seven bacterial species as a percentage of total bacterial 16S rRNA was performed with the ABI PRISM 7700 Sequence Detection System (AB Applied Biosystems, Life Technologies Corp.) using the same primers as in Table 3.2. Quantification of DNA for each bacterial species in rumen contents was performed with Quantifast Kit (Qiagen) using SYBR green chemistry. Standards and samples were assayed in 25 µl reaction mixture containing 15 µl of Quantifast SYBR Green Master Mix, 8 µl of nuclease-free water and 2 µl of DNA template. Amplifications were performed under the following fast conditions: 95°C for 5 min, 95°C for 10 s and a 30 s annealing/elongation for 40 cycles at the temperature for each primer pair reported in Table 3.2. The melting curve of PCR products was monitored by slow heating with an increment of 0.1°C s^{-1} from 60 – 95°C with fluorescence collection at 0.1°C intervals to confirm specificity of amplification. Quantification was done as previously described (Chapter 3.3.3) by the creation of a standard curve for each

bacterial species and the calculation of copy numbers in 20 ng of DNA was done using previously stated equation (Chapter 3.3.3).

Amplification products were verified by horizontal gel electrophoresis of a 5 µl aliquot in a 1% agarose gel in Tris-Acetate-EDTA (40 mM Tris acetate, 1mM EDTA; pH 8.5), followed by ethidium bromide staining and visualisation under UV light. A 1 kb Ladder (Quickload, New England Biolabs Ltd., Pickering, ON, Canada.) was included on each gel to enable confirmation of the size of amplified product. To minimize errors of absolute quantification of DNA from rumen samples, relative quantification methods were used as previously reported (Chapter 3.3.3). A non-degenerate, domain-level primer set that amplified all bacterial species was used as the reference primer set (Table 3.2).

4.2.7 Pyrosequencing Analysis

Pyrosequencing analysis of the V1-V3 region of 16S rRNA on 36 samples yielded 126,964 raw reads. Reads with an average quality score of less than 35, homopolymers greater than eight bases, and sequences with one or more ambiguous bases were removed from the dataset. Sequences were then aligned against the SILVA alignment database for 16S rRNA genes to define operational taxonomic units (OTUs) (Schloss *et al.* , 2009). Sequences that did not span the longest alignment region were also removed from the dataset. Sequences were trimmed so that reads overlapped in the same alignment space (Schloss *et al.* , 2009), producing read lengths ranging from 167 to 349 bps all starting at an optimized starting point. Pyrosequencing noise due to base call errors was minimized in the dataset using the pre-cluster algorithm in MOTHUR (Huse *et al.* , 2010), whereby rare sequences highly similar to abundant sequences were re-classified as their abundant homologue. Chimeras were removed from the samples using the

sequence collection (UCHIME) as its own reference database (Edgar *et al.* , 2011). A distance matrix was constructed using the average neighbor algorithm at 0.03 (equivalent to species), 0.05 (genus), 0.25 (phylum) phylogenetic distances. Pairwise distances between aligned sequences were calculated at a 0.97% similarity cutoff to optimize potential similarities and then clustered into unique OTUs. In total, there were 9,323 high quality reads with an average of 4,267 ± 1419 reads and 149 ± 28 unique OTUs per individual sample. MOTHUR was also used to generate rarefaction curves, species richness using Chao1 and abundance-based coverage estimation (ACE), species diversity with Shannon-Weiner and Simpsons indices, and to create a dendrogram based on treatment differences using the Jaccard index (Table 4.2). Calculations of percentage of sequences within taxonomic classifications at the genus and species level were performed using a custom summation script.

4.2.8 Statistical Analysis

Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 5.1, Applied Maths, Inc., Austin, TX, U.S.A) to create similarity matrices to identify community population differences among treatments and individual animals. Bands were visually selected based on peak height. Using average Dice's similarity coefficient (D_{sc}) index, with an optimization of 1.0% and with a tolerance of 1.0%, clustering was carried out using the unweighted pair group method with arithmetic means (UPGMA). Read number, sample coverage, unique OTUs, sample richness (Chao1 and ACE) and sample diversity (Shannon-Weiner and Simpson's indices) were compared with one-way ANOVA using the Proc Mixed procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA). Using the same procedure,

Table 4.2. Summary of dietary treatment comparisons for unique OTUs, richness estimates, and diversity indices. The minimum number of unique OTUs in each population was determined with a 10 % difference level.

	Forage	Mixed Forage	High Grain	Acidosis Challenge	Challenge Recovery	SEM	*P*-value
Number of sequences	5045	3731	4349	4148	4389	579	0.68
Coverage (%)	99.0	98.8	99.1	98.9	99.0	0.2	0.64
Total # of unique OTUs	161	147	144	148	149	11	0.90
Richness estimate							
Chao1	198	181	183	192	181	16	0.94
ACE	192	180	179	192	189	15	0.94
Diversity indices							
Shannon-Weiner	3.72	3.36	3.31	3.43	3.19	0.14	0.12
Simpson's	0.95	0.92	0.91	0.93	0.89	0.02	0.16

real-time PCR relative quantification and rumen fermentation variables including VFA and pH were analyzed for effect of treatment, animal and interaction between animal and treatment. Percent taxonomic data based on sequence analysis were similarly analyzed after first being log-transformed (Duval *et al.* , 2007). Means were separated using Tukey's honest significant difference (HSD). All pH variables were additionally analyzed in a pairwise correlation to all unique OTUs. Significance was declared at $P \leq 0.05$; trends were indicated at $P \leq 0.10$.

4.3 Results

4.3.1 Diversity and Richness Analysis

Rarefaction curves provide a way of comparing the richness observed in different samples and determine the extent of sampling relative to how much sampling is needed to accurately describe the microbial community. All treatments showed similar rarefaction curves with levels tending to plateau after about 15,000 sequences (Figure 4.1). This indicates that the observed level of richness, as determined by the unique sequences and overall sampling intensity, was sufficient to accurately describe the richness of the rumen epimural microbial ecology across sampling periods. The ACE and Chao1 estimates were calculated to compare species richness which is defined as the total number of species among samples, by estimating the minimum number of unique OTUs at each sampling point (Table 4.2). The number of OTUs that were unique to each treatment ranged from 144 to 161. Chao1 and ACE values both showed species richness to be the highest during the forage and the acidotic challenge periods; however, there was no statistical difference among diets. Similarly, the Shannon-Weiner index and the Simpson indices both showed an overall high level of diversity in the rumen epimural community. However, no treatment differences were noted in terms of diversity and richness (Table 4.2). The Shannon-Weiner and Simpson's diversity indices were calculated to determine the diversity of

OTUs within each treatment, defined as the number of species and their relative abundance in the overall epimural community (Martin, 2002). Lack of dietary treatment differences shows the rich diversity of this ecosystem regardless of environmental parameters.

4.3.2 Cluster Analysis

A Jaccard cluster analysis of sequence data showed that each epithelial population possessed unique OTUs (Figure 4.2). Samples grouped according to diet composition with those diets that contained high levels of forage (forage, mixed forage) being more closely related than those collected when heifers were fed high grain prior to, during and after the acidotic challenge. Sub-clusteringof samples collected during the high grain and acidotic challenge points as compared to the challenge recovery was also evident (Figure 4.2). The epithelial samples collected during the high grain and acidotic challenge periods showed the highest species similarities. However, even with these similarities, these two treatments had distinct populations that differed from each other as well as from the other diets.

4.3.3 PCR-DGGE and Real-Time PCR

Polymerase chain reaction-DGGE had a total number of bands per sample ranging from 8 to 31 with an average of 21 bands per sample based on Bionumerics peak analysis with visual adjustments. No clustering was observed (Figure 4.3), which is partially due to the low band numbers in some samples resulting in separate clustering. However, heifer 143 showed the most significant clustering with 89% Dice similarity between the high grain and acidotic samples and mixed forage samples sharing 87% similarity to those treatments.

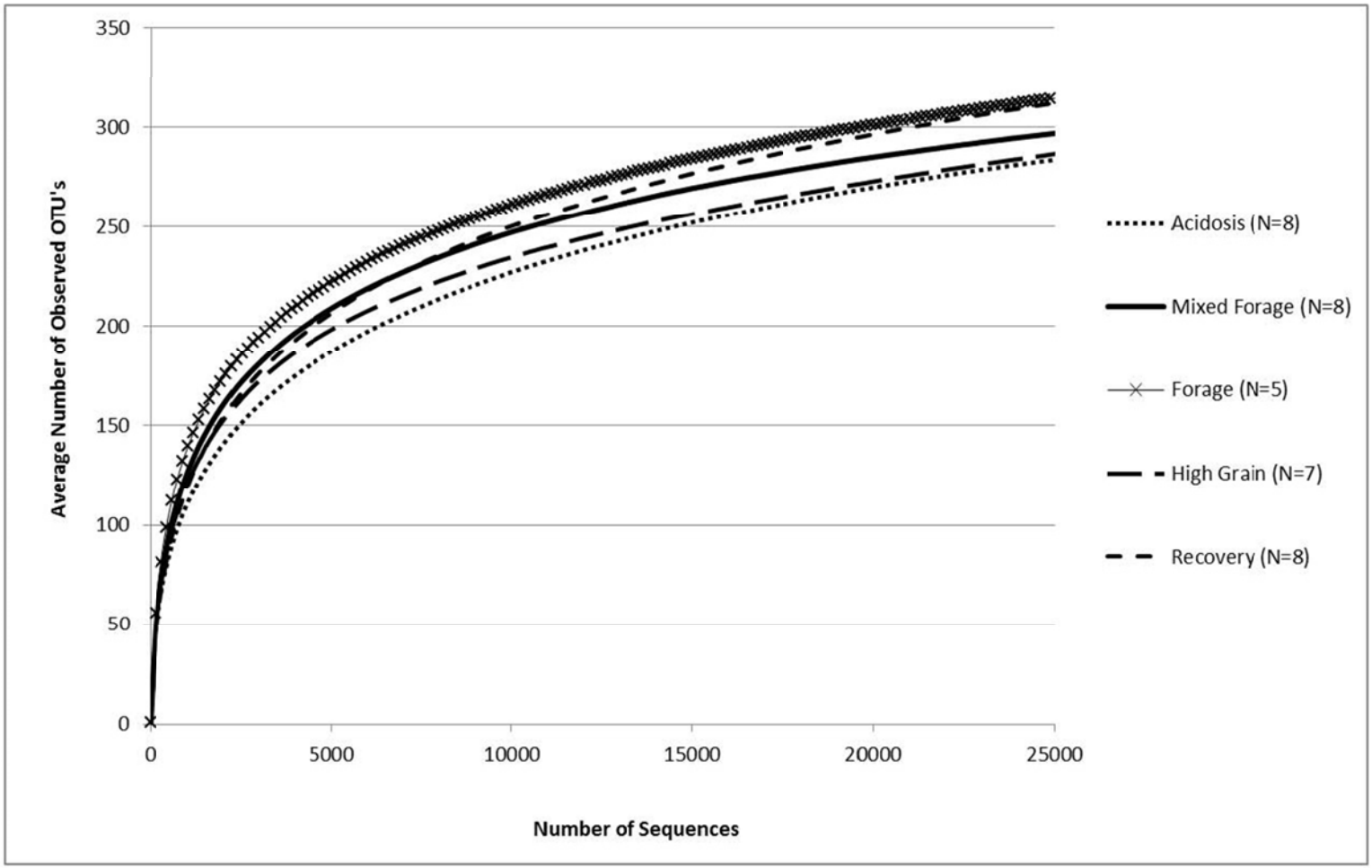


Figure 4.1. Rarefaction curves for rumen epithelial bacterial communities for each heifer treatment group. Each curve represents t treatment average based on 5 to 8 heifers in each group. Unique OTU's are estimated at a 10% difference level.

A low level of similarity (< 90%) among heifers and treatment groups indicates a high degree of diversity between the dietary treatments as well as between individual heifers. Of the six bacterial species evaluated in this study only two, *Ruminococcus* and *Prevotella* spp. were detected in all heifers regardless of diet (Table 4.3). Quantities of prominent ruminal bacteria expressed as a percentage of total enumerated bacteria ranged from 0.0 to 6.74% among diets (Table 4.3) and from 0.0 to 8.52% among individual heifers (Table 4.4). When comparing the relative abundance of the *F. succinogenes* populations (P=0.01) among individual heifers, heifers 7 and 41 possessed no detectable *F. succinogenes* whereas 156 had the highest (P=0.01) relative abundance of this bacterium (1.42%; Table 4.4). When diets were compared, only *F. succinogenes* varied (P=0.007) across diets with the highest proportion (1.25%) occurring in cattle fed the forage diet whereas this bacterium was not detected in the high grain treatment (0.0%). *Selenomonas ruminantium* tended to be higher (P=0.09) during the acidotic challenge and recovery period as compared to the other diets (Table 4.4).

4.3.4 Percent Taxonomic Estimates

Butyrivibrio was more ($P < 0.001$) abundant when heifers were fed forage and mixed forage diets as compared to samples collected during the acidotic challenge (Table 4.5). *Prevotella* tended ($P = 0.08$) to be highest in the high grain, acidotic challenge and recovery samples as compared to the forage and mixed samples. A total of 38 different genera were found to be influenced ($P \leq 0.05$) by dietary treatment with 14 of these being most abundant in samples collected during the acidotic challenge. These included both cultured and uncultured genera including *Adhufec269, Atopobium, Desulfocurvus, Fervidicola, IS cTPY-17 adhufec52, IS Eub cellulosolvens Lactobacillus, OlsenellaRC39, Roseburia, Sharpea,*

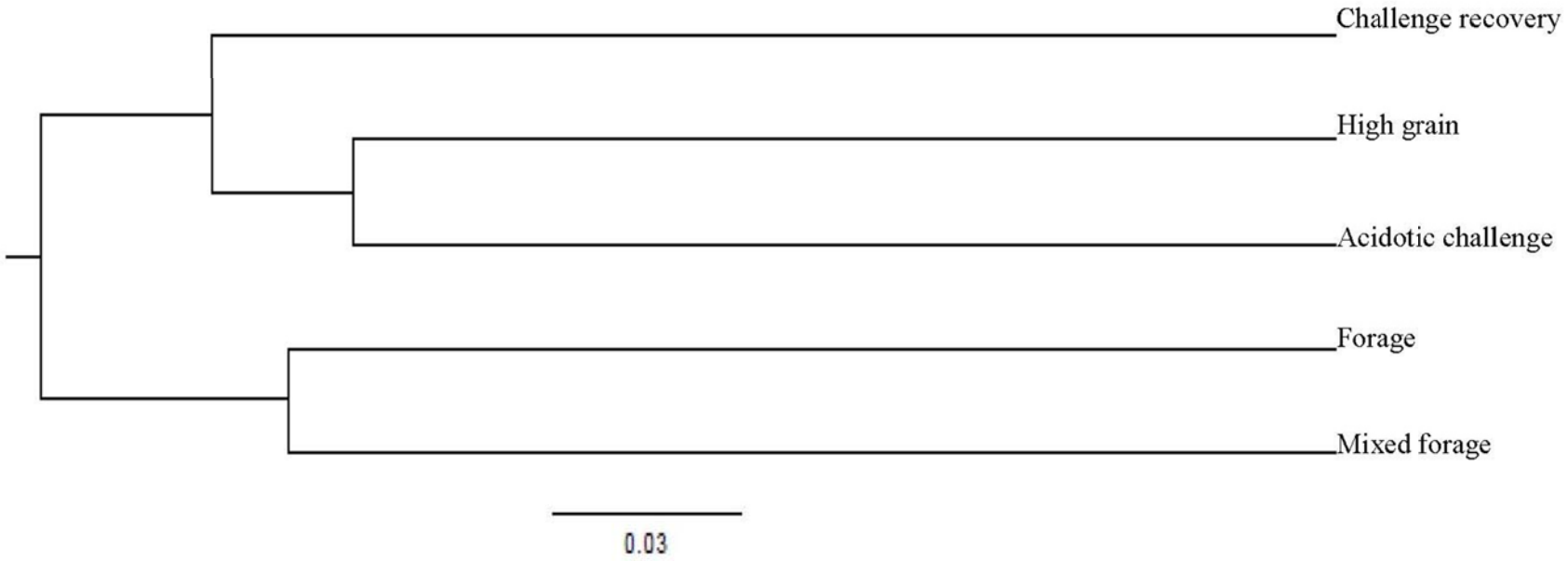


Figure 4.2. Cluster analysis of dietary treatments created using Jaccard analysis to show dissimilarity between epithelial populations based on unique operational taxonomic units (OTUs) for each treatment. OTUs are estimated at a 10% difference level.

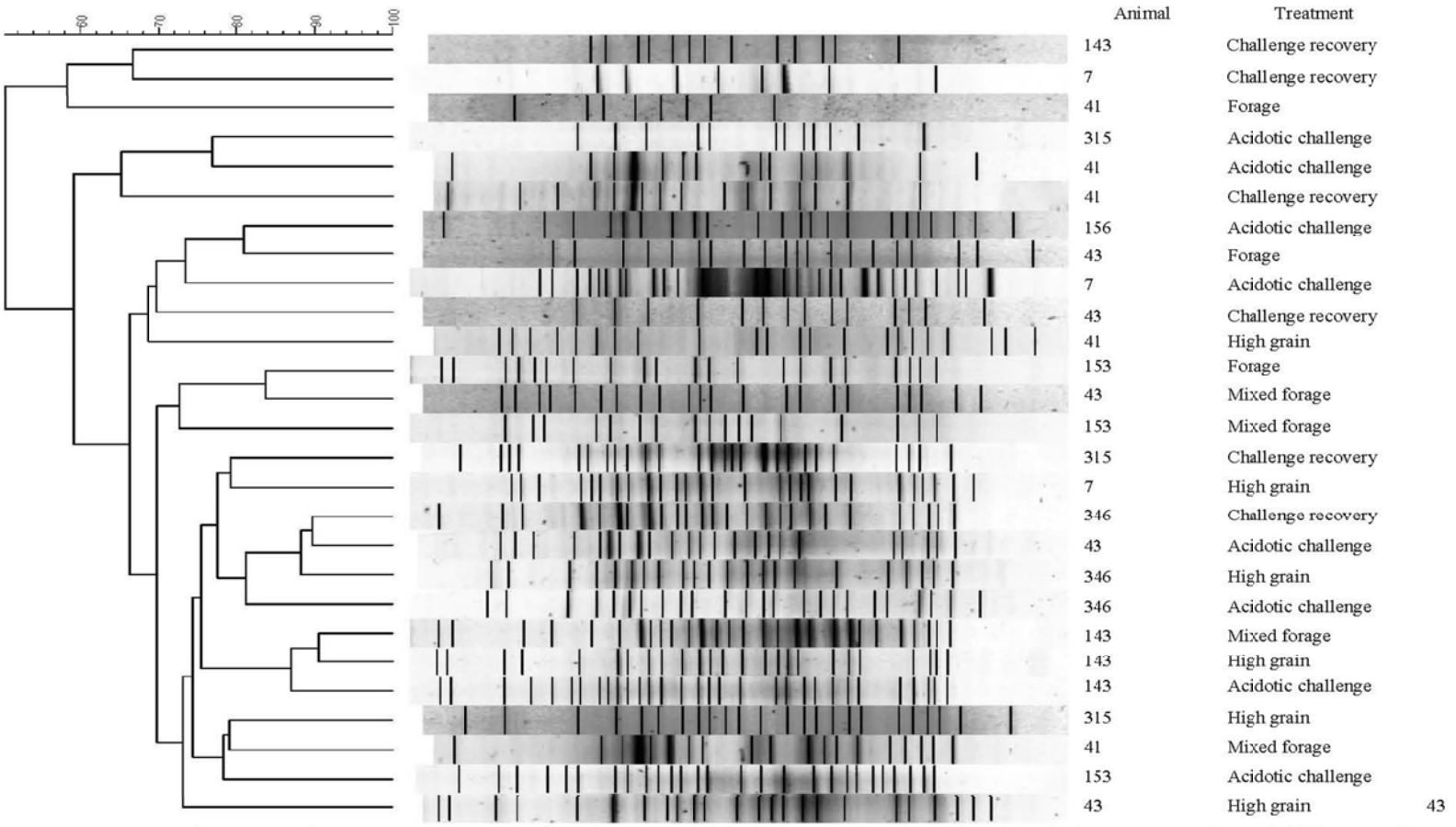


Figure 4.3. Dendrogram of PCR-DGGE analysis of rumen epithelial samples from heifers fed a progression of different diets (Forage, Mixed, High Grain, Acidosis Challenge and Challenge Recovery). Clustered with Dice (Opt: 1.0%) (Tol: 1.0%) and UPGMA

Solobacterium, Succiniclastium and *Succinivibrio. Desulfocurvus* was found exclusively during the acidotic challenge, where they accounted for nearly 1% of total rumen epithelial bacteria. When comparing among heifers, 11 distinct genera differed ($P \leq 0.05$) among individual heifers (Table 4.6).Genera *Atopobacter*, *F24-B10*, and *U29-B03* were found primarily in only one of the 8 heifers regardless of the period that the ruminal sample was collected (Table 4.6). A correlation analysis of all pH variables to all identifiable epithelial genera was performed and 43 genera were found to be correlated to one or more pH variables (Table 4.7).

Thermodesulfobium was the only genera that correlated to all pH variables, with associated reads been reduced at low pH (Table 4.7). All genera that showed a decrease in total abundance as the minimum daily pH decreased also decreased in total abundance as the pH duration and area under all pH benchmarks increased. Other genera that responded with decreased abundance as the minimum daily pH decreased included: *Azonexus*, *Butyrivibrio, Carboxydibrachium, Eubacteria, Fervidoicola, Fusobacterium, Incertae Sedis C. viride, Marvinbryantia, RC1-13, RF21, RF38, RFN8-YE57, Ruminococcus 1, Saccharofermentans, and vadinHS42*. Additionally, those genera that were determined to be part of an "Uncultured/Unclassified" grouping similarly showed sensitivity to low pH, decreasing in total abundance with lower pH. Conversely, 16 genera increased in percent abundance with more acidic rumen conditions: *Anerophaga, Atopobium, cc142, IS Butryivibrio, IS cTPY 17 adhufec 52, IS R. gnavus, Lactobacillus, Megasphaera, Mitsuokella, Olsenella, RC39, Selenomonas, Solobacterium, Streptococcus, Succiniclastium,* and *Succinivibrio* (Table 4.7). All genera that increased with lower daily pH, also increased as duration and area under pH benchmarks 5.8, 5.5 and 5.2 increased. *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the most abundant phyla and their percentage of abundance remained

Table 4.3. Effect of individual heifer on the percent (%) of total enumerated epithelial bacteria 16S rRNA genes of dominant rumen bacterial species using quantitative real-time PCR.

	Individual animal									
Bacteria	7	41	43	143	153	156	315	346	SEM	*P*-value
Ruminococcus spp.	2.60	4.50	2.12	7.28	1.36	8.52	6.85	1.74	2.309	0.197
Fibrobacter succinogenes	0.00a	0.00a	0.41ab	0.82ab	0.19a	1.42b	0.48ab	0.51ab	0.269	0.011
Prevotella spp.	1.53	3.93	2.02	4.62	1.32	4.25	6.23	1.16	1.664	0.370
Selenomonas ruminantium	1.11	5.32	0.85	3.49	0.00	3.26	6.84	0.69	2.331	0.372
Megasphaera elsdenii	0.56	2.57	0.02	0.20	6.23	0.20	0.16	0.18	1.069	0.602
Streptococcus bovis	0.66	0.57	0.75	4.24	0.00	0.01	0.00	0.00	1.423	0.404

Table 4.4. Effect of diet on the percent (%) of total enumerated epithelial bacteria 16S rRNA genes of dominant rumen bacterial species using quantitative real-time PCR.

	Dietary Treatment						
Bacteria	Forage	Mixed forage	High grain	Acidotic challenge	Challenge recovery	SEM	*P*-value
Ruminococcus spp.	5.48	4.46	2.19	2.99	6.74	1.829	0.369
Fibrobacter succinogenes	1.25b	0.63ab	0.00a	0.88a	0.45a	0.214	0.007
Prevotella spp.	1.93	1.34	2.83	3.32	6.24	1.318	0.086
Selenomonas ruminantium	2.42	0.38	0.92	4.73	4.11	1.851	0.362
Megasphaera elsdenii	0.24	0.00	0.27	2.25	0.84	0.847	0.252
Streptococcus bovis	0.28	0.00	0.00	3.33	0.29	1.127	0.157

Table 4.5. Percent contribution of taxon/genus level epithelial taxa to the rumen microbial populations compared between Forage, Mixed Forage, High Grain, Acidotic Challenge and Challenge Recovery diets. Remaining genera not shown due to non-significant differences among treatments.

	Treatment						
Percent taxon	Forage	Mixed forage	High grain	Acidotic challenge	Challenge recovery	SEM	*P*-value
Adhufec269	0.00a	0.07a	0.56b	0.76b	0.69b	0.07	0.06
Anaerovorax	0.49ab	0.99b	0.14a	0.07a	0.27ab	0.07	0.01
Atopobium	0.00a	0.00a	0.93bc	1.51c	0.44ab	0.10	<0.001
Azonexus	0.51b	0.61b	0.00a	0.00a	0.09a	0.04	0.01
Butyrivibrio	8.63bc	9.66c	5.14ab	2.42ab	2.40a	0.90	<0.001
cc142	0.57a	0.74a	2.34b	2.50b	2.55b	0.30	0.04
Coprobacillus	0.44b	0.00a	0.00a	0.00a	0.00a	0.01	0.01
Desulfocurvus	0.00a	0.00a	0.00a	0.74b	0.00a	0.03	0.01
Faecalibacterium	0.36b	0.00a	0.00a	0.00a	0.00a	0.01	<0.001
Fervidicola	0.00a	0.00a	0.00a	0.33b	0.08a	0.02	0.05
Filifactor	0.57b	0.00a	0.00a	0.00a	0.00a	0.01	0.01
Fusobacterium	1.06b	0.00a	0.00a	0.00a	0.00a	0.03	<0.001
IS cTPY-17_adhufec52	0.00a	0.00a	0.32b	0.54b	0.11b	0.03	0.03
IS Eub. Cellulosolvens	0.00a	0.00a	0.16b	0.30b	0.19b	0.02	0.07
Lactobacillus	0.00a	0.00a	0.00a	4.48b	0.00a	0.17	0.07
Marvinbryantia	0.16b	0.40b	0.00a	0.00a	0.00a	0.02	0.02
Mogibacterium	3.36a	6.43b	8.07b	4.61ab	4.65ab	0.92	0.07
Olsenella	0.00a	0.00a	0.09a	1.60b	0.14a	0.07	<0.001
Oxobacter	1.35b	0.36a	0.67ab	0.21a	0.83ab	0.10	0.04
Porphyromonas	0.76b	0.00a	0.00a	0.00a	0.00a	0.02	<0.001
Prevotella	2.62a	1.86a	4.21b	4.19b	6.74b	0.67	0.08
Proteiniborus	0.36b	0.00a	0.00a	0.06b	0.00a	0.01	0.02
RC1-13	0.53ab	1.26b	0.67ab	0.34a	0.48a	0.11	0.01
RC25	0.46b	0.28b	0.00a	0.00a	0.00a	0.02	0.03
RC39	0.00a	0.00a	1.50b	2.93c	0.83ab	0.19	<0.001
RF21	1.20a	3.13b	1.86ab	0.91a	1.43ab	0.29	0.01
RF38	0.49b	0.26ab	0.00a	0.00a	0.00a	0.02	<0.001
Roseburia	0.00a	0.00a	0.88b	0.98b	0.92b	0.10	0.03
Saccharofermentans	1.25b	1.19b	0.22a	0.00a	0.09a	0.08	<0.001
Sharpea	0.00a	0.00a	0.20b	1.00b	0.00a	0.04	0.06
Solobacterium	0.12a	0.23a	1.68ab	3.48b	2.08ab	0.27	<0.001
Sporobacter	0.62b	0.07a	0.43ab	0.20ab	0.61b	0.06	0.07
Succiniclasticum	0.44a	0.39a	3.20c	2.71bc	1.26ab	0.28	<0.001
Succinivibrio	0.00a	0.00a	0.39b	0.56b	0.00a	0.03	0.03
Syntrophococcus	0.29a	0.69ab	0.98ab	1.16b	1.20b	0.15	0.02
Thermodesulfobium	0.60ab	1.13b	0.34ab	0.09a	0.10a	0.07	0.01
Thermohalobacter	0.62b	0.07a	0.00a	0.07a	0.00a	0.02	<0.001
Uncultured	1.98bc	2.14b	1.14ab	0.68a	0.73a	0.21	<0.001

Table 4.6. Percent contribution of epithelial taxa to the rumen microbial populations averaged for Forage, Mixed Forage, High Grain, Acidosis Challenge and Challenge Recovery diets for individual heifers. Remaining taxa not shown due to non-significant differences among diets.

	Individual animals									
Taxa	7	41	43	143	153	156	315	346	SEM	*P*-Value
12-18	0.0	0.0	0.0	0.0	1.6	2.5	8.1	1.2	0.7	0.07
adhufec405	2.6	0.0	1.1	0.0	0.0	2.3	8.1	4.5	0.8	0.11
Atopobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.2	0.06
Carboxydibrachium	0.0	1.1	0.0	2.6	6.3	0.0	0.0	1.4	0.6	0.11
Comamonas	15.9a	5.6a	38.1b	40.8b	29.8b	34.2b	35.4b	29.4b	3.1	0.04
Dialister	0.0	1.3	1.3	0.0	1.4	3.5	10.6	3.2	0.9	0.07
F24-B10	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	5.4b	0.0a	0.3	<0.001
Guggenheimella	4.3ab	2.7a	6.8ab	10.9b	5.7ab	11.8b	2.8ab	9.4ab	0.8	0.03
IS Eub. cellulosolvens	5.3	2.1	0.0	0.0	1.3	0.0	0.0	1.4	0.5	0.09
Oxobacter	11.4	5.7	11.3	5.2	1.4	2.8	0.0	9.6	1.2	0.06
p-4496-6Wb3	2.5ab	0.0a	1.6ab	9.7b	2.2ab	0.0a	5.7ab	2.5ab	0.7	0.02
Pelospora	15.4ab	15.2ab	15.8ab	31.6b	3.2a	21.2ab	11.1ab	10.4ab	2.0	0.03
rc1-13	4.2	2.9	5.2	13.4	8.5	4.3	12.5	3.9	1.1	0.07
RC20	0.0a	0.0a	0.0a	0.0a	7.0b	0.0a	0.0a	1.4ab	0.6	0.01
Succinivibrio	0.0	0.0	1.3	0.0	0.0	3.1	9.2	3.9	0.8	0.07
Thermotalea	0.0a	4.9b	4.1b	4.0b	4.2b	0.0a	0.0a	0.0a	0.6	0.05
U29-B03	0.0a	0.0a	0.0a	0.0a	0.0a	3.5b	0.0a	0.0a	0.2	<0.001
uncultured	9.4ab	8.6ab	13.3b	4.2a	16.5b	4.3a	2.7a	10.9ab	1.2	0.05
Verminephrobacter	0.0a	0.0a	0.0a	6.4b	1.9ab	0.0a	1.8ab	4.2b	0.6	0.03
wet75	9.1ab	3.1a	7.5ab	11.4ab	11.8ab	19.01b	2.5a	7.8ab	1.2	0.05

Letters in each row indicate significant difference between treatments.

Table 4.7. Correlation of all calculated pH variables from the Acidotic Challenge treatment to epithelial taxa. Only those taxa found to be significant are shown. Acidotic Challenge pH variables are the mean for all heifers on that diet.

		pH variables								
Taxa		pH min	pH mean	pH max	Duration under pH 5.8 (min)	pH area under 5.8 (pH×min)	Duration under pH 5.5 (min)	pH area under 5.5 (pH×min)	Duration under pH 5.2 (min)	pH area under 5.2 (pH×min)
Acetitomaculum	Correlation	-0.32	-0.31	0.19	0.20	0.45	0.24	0.52	0.24	0.57
	P-Value	0.09	0.10	0.32	0.29	0.01	0.20	0.00	0.22	0.00
Acidaminococcus	Correlation	-0.32	-0.33	0.16	0.27	0.39	0.26	0.42	0.24	0.42
	P-Value	0.09	0.08	0.41	0.16	0.04	0.17	0.03	0.21	0.02
Anaerophaga	Correlation	-0.38	-0.61	0.12	0.25	0.62	0.32	0.71	0.41	0.80
	P-Value	0.04	0.00	0.52	0.18	0.00	0.09	0.00	0.03	0.00
Anaerovorax	Correlation	0.34	0.44	0.12	-0.43	-0.40	-0.45	-0.36	-0.38	-0.31
	P-Value	0.07	0.02	0.53	0.02	0.03	0.02	0.05	0.04	0.11
Atopobacter	Correlation	-0.30	-0.17	0.16	0.38	0.30	0.36	0.25	0.24	0.14
	P-Value	0.11	0.39	0.41	0.04	0.11	0.06	0.19	0.20	0.47
Atopobium	Correlation	-0.61	-0.58	-0.37	0.76	0.61	0.73	0.51	0.60	0.35
	P-Value	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.06
Azonexus	Correlation	0.39	0.40	-0.06	-0.50	-0.39	-0.48	-0.32	-0.43	-0.25
	P-Value	0.04	0.03	0.75	0.01	0.04	0.01	0.09	0.02	0.18
Bacteroides	Correlation	-0.11	-0.34	-0.07	0.07	0.29	0.13	0.35	0.20	0.39
	P-Value	0.57	0.08	0.72	0.72	0.13	0.52	0.06	0.30	0.03
Butyrivibrio	Correlation	0.55	0.49	0.20	-0.59	-0.52	-0.57	-0.46	-0.51	-0.38
	P-Value	0.00	0.01	0.31	0.00	0.00	0.00	0.01	0.00	0.04
Carboxydibrachium	Correlation	0.40	0.27	0.26	-0.34	-0.27	-0.34	-0.23	-0.30	-0.16
	P-Value	0.03	0.15	0.18	0.07	0.15	0.07	0.23	0.11	0.40
cc142	Correlation	-0.43	-0.26	-0.17	0.43	0.24	0.38	0.15	0.31	0.03
	P-Value	0.02	0.18	0.38	0.02	0.21	0.04	0.43	0.11	0.87
Comamonas	Correlation	0.17	0.17	-0.06	-0.07	-0.30	-0.20	-0.36	-0.27	-0.39
	P-Value	0.38	0.37	0.78	0.71	0.11	0.29	0.06	0.16	0.04
Dialister	Correlation	-0.32	-0.36	0.06	0.37	0.34	0.36	0.31	0.39	0.28
	P-Value	0.09	0.06	0.74	0.05	0.07	0.05	0.11	0.03	0.13
Eub. brachy	Correlation	0.48	0.35	0.00	-0.37	-0.26	-0.31	-0.22	-0.28	-0.17
	P-Value	0.01	0.06	0.99	0.05	0.17	0.10	0.26	0.14	0.37
Fervidicola	Correlation	0.43	0.34	-0.06	-0.33	-0.28	-0.34	-0.24	-0.35	-0.18
	P-Value	0.02	0.07	0.75	0.08	0.15	0.08	0.22	0.06	0.35

Taxa		pH variables								
		pH min	pH mean	pH max	Duration under pH 5.8 (min)	pH area under 5.8 (pH×min)	Duration under pH 5.5 (min)	pH area under 5.5 (pH×min)	Duration under pH 5.2 (min)	pH area under 5.2 (pH×min)
Fusobacterium	Correlation	0.48	0.37	0.61	-0.31	-0.25	-0.27	-0.22	-0.21	-0.18
	P-Value	0.01	0.05	0.00	0.10	0.19	0.16	0.26	0.28	0.36
Incertae Sedis C. viride	Correlation	0.47	0.33	-0.06	-0.35	-0.24	-0.29	-0.20	-0.27	-0.16
	P-Value	0.01	0.08	0.76	0.06	0.20	0.12	0.31	0.15	0.42
IS Butyrivibrio	Correlation	-0.36	-0.54	0.11	0.34	0.56	0.36	0.61	0.47	0.66
	P-Value	0.05	0.00	0.55	0.07	0.00	0.05	0.00	0.01	0.00
IS cTPY-17 adhufec52	Correlation	-0.49	-0.45	-0.04	0.33	0.47	0.40	0.48	0.55	0.54
	P-Value	0.01	0.01	0.84	0.08	0.01	0.03	0.01	0.00	0.00
IS R. gnavus	Correlation	-0.38	-0.61	0.12	0.25	0.62	0.32	0.71	0.41	0.80
	P-Value	0.04	0.00	0.52	0.18	0.00	0.09	0.00	0.03	0.00
Lactobacillus	Correlation	-0.57	-0.66	0.09	0.36	0.74	0.43	0.84	0.53	0.93
	P-Value	0.00	0.00	0.64	0.05	0.00	0.02	0.00	0.00	0.00
Marvinbryantia	Correlation	0.45	0.42	-0.15	-0.45	-0.40	-0.45	-0.36	-0.41	-0.30
	P-Value	0.01	0.02	0.44	0.01	0.03	0.01	0.06	0.03	0.12
Megasphaera	Correlation	-0.52	-0.63	0.09	0.33	0.72	0.40	0.82	0.48	0.90
	P-Value	0.00	0.00	0.64	0.08	0.00	0.03	0.00	0.01	0.00
Mitsuokella	Correlation	-0.38	-0.61	0.12	0.25	0.62	0.32	0.71	0.41	0.80
	P-Value	0.04	0.00	0.52	0.18	0.00	0.09	0.00	0.03	0.00
Olsenella	Correlation	-0.61	-0.51	-0.05	0.41	0.58	0.45	0.61	0.57	0.67
	P-Value	0.00	0.00	0.79	0.03	0.00	0.01	0.00	0.00	0.00
rc1-13	Correlation	0.49	0.50	-0.09	-0.50	-0.52	-0.54	-0.49	-0.51	-0.42
	P-Value	0.01	0.01	0.63	0.01	0.00	0.00	0.01	0.01	0.02
RC25	Correlation	0.35	0.30	-0.29	-0.39	-0.32	-0.39	-0.26	-0.36	-0.21
	P-Value	0.06	0.11	0.13	0.03	0.09	0.04	0.17	0.05	0.28
RC39	Correlation	-0.75	-0.71	-0.08	0.64	0.79	0.69	0.79	0.70	0.77
	P-Value	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00
RF21	Correlation	0.44	0.45	-0.02	-0.35	-0.35	-0.31	-0.34	-0.29	-0.34
	P-Value	0.02	0.01	0.91	0.07	0.06	0.10	0.07	0.13	0.07
RF38	Correlation	0.52	0.38	0.08	-0.43	-0.35	-0.41	-0.30	-0.35	-0.24
	P-Value	0.00	0.04	0.67	0.02	0.06	0.03	0.11	0.06	0.20
RFN71	Correlation	0.30	0.35	-0.36	-0.36	-0.42	-0.44	-0.40	-0.50	-0.35
	P-Value	0.12	0.06	0.06	0.05	0.02	0.02	0.03	0.01	0.07

Taxa		pH variables								
		pH min	pH mean	pH max	Duration under pH 5.8 (min)	pH area under 5.8 (pH×min)	Duration under pH 5.5 (min)	pH area under 5.5 (pH×min)	Duration under pH 5.2 (min)	pH area under 5.2 (pH×min)
RFN8-YE57	Correlation	0.42	0.42	0.06	-0.41	-0.42	-0.42	-0.40	-0.38	-0.32
	P-Value	0.02	0.02	0.77	0.03	0.02	0.02	0.03	0.04	0.09
Ruminococcus 1	Correlation	0.43	0.46	0.07	-0.42	-0.40	-0.42	-0.37	-0.42	-0.32
	P-Value	0.02	0.01	0.73	0.02	0.03	0.02	0.05	0.02	0.09
Saccharofermentans	Correlation	0.61	0.52	-0.04	-0.59	-0.51	-0.58	-0.44	-0.50	-0.36
	P-Value	0.00	0.00	0.86	0.00	0.01	0.00	0.02	0.01	0.05
Selenomonas	Correlation	-0.38	-0.61	0.12	0.25	0.62	0.32	0.71	0.41	0.80
	P-Value	0.04	0.00	0.52	0.18	0.00	0.09	0.00	0.03	0.00
Solobacterium	Correlation	-0.63	-0.59	-0.01	0.55	0.57	0.60	0.52	0.62	0.46
	P-Value	0.00	0.00	0.97	0.00	0.00	0.00	0.00	0.00	0.01
Streptococcus	Correlation	-0.43	-0.63	0.12	0.28	0.66	0.35	0.76	0.44	0.85
	P-Value	0.02	0.00	0.55	0.14	0.00	0.06	0.00	0.02	0.00
Succiniclasticum	Correlation	-0.43	-0.42	-0.29	0.60	0.47	0.57	0.39	0.42	0.27
	P-Value	0.02	0.02	0.12	0.00	0.01	0.00	0.04	0.02	0.16
Succinivibrio	Correlation	-0.41	-0.43	-0.07	0.49	0.39	0.53	0.32	0.61	0.23
	P-Value	0.03	0.02	0.74	0.01	0.04	0.00	0.10	0.00	0.23
Thermodesulfobium	Correlation	0.58	0.49	0.44	-0.51	-0.48	-0.52	-0.43	-0.49	-0.36
	P-Value	0.00	0.01	0.02	0.00	0.01	0.00	0.02	0.01	0.05
uncultured	Correlation	0.56	0.60	0.04	-0.48	-0.53	-0.49	-0.52	-0.52	-0.51
	P-Value	0.00	0.00	0.83	0.01	0.00	0.01	0.00	0.00	0.00
vadinHA42	Correlation	0.40	0.37	0.08	-0.28	-0.35	-0.32	-0.35	-0.34	-0.31
	P-Value	0.03	0.05	0.69	0.14	0.06	0.10	0.06	0.08	0.10
wet75	Correlation	0.22	0.20	-0.17	-0.05	-0.32	-0.22	-0.38	-0.38	-0.41
	P-Value	0.25	0.30	0.37	0.81	0.09	0.26	0.04	0.04	0.03

Letters in each row indicate significant difference between treatments.

Table 4.8. Percent contribution of phyla level epithelial taxa to the rumen microbial populations averaged over individual heifers. Forage, Mixed Forage, High Grain, Acidosis Challenge and Challenge Recovery for individual heifers.

	Treatment						
Phyla	Forage	Mixed forage	High grain	Acidosis Challenge	Challenge Recovery	SEM	*P*-Value
Actinobacteria	2.05a	1.89a	3.39ab	4.93b	2.60a	0.51	0.0001
Bacteroidetes	9.86	7.87	9.05	8.42	12.81	1.60	0.34
Candidate division TM7	1.58c	1.30bc	0.26a	0.46ab	0.28a	0.12	0.0003
Chloroflexi	0.00	0.00	0.00	0.09	0.00	0.00	0.49
Fibrobacters	0.44	0.11	0.16	0.16	0.07	0.03	0.63
Firmicutes	66.88	73.65	74.67	74.56	72.03	12.12	0.11
Fusobacteria	2.58b	0.11a	0.10a	0.00a	0.00a	0.07	0.01
Planctomycetes	0.30	0.31	0.00	0.00	0.00	0.02	0.36
*Proteobacteria**	13.36	12.28	9.99	9.91	9.83	1.82	0.17
Spirochaetes	0.59	0.74	0.50	0.06	0.52	0.08	0.37
Synergistetes	0.17ab	0.10ab	0.63b	0.08a	0.25ab	0.04	0.05
Tenericutes	0.92b	0.38ab	0.11ab	0.00a	0.14ab	0.04	0.05

*Significant animal effect noted for *Proteobacteria* (P=0.04)

Letters in each row indicate significant difference between treatments.

relatively constant despite changes in diet over the duration of the experiment (Table 4.8). Forage and mixed forage diets exhibited increased ($P \leq 0.05$) levels of *Candidate division TM7*, *Fusobacterium* and *Tenericutes*. Comparatively, samples from heifers fed the high grain diet showed the highest levels ($P = 0.05$) of *Synergistetes;* whereas *Actinobacteria* was highest ($P < 0.001$) in heifers during the period of acidotic challenge (Table 4.8). All epithelial phyla were also used in a correlation analysis to all pH variables and 5 phyla were also found to be correlated to one or more pH variables (Table 4.9). *Actinobacteria* was negatively correlated to lowest daily pH and mean pH, but positively correlated to duration and area under all pH benchmarks. *Candidate division TM7, Planctomycetes* and *Tenericutes* were all positively correlated to pH nadir and mean pH while being negatively correlated to all other pH measures (Table 4.9).

4.3.5 Core Microbiome

The core bacterial community shared by all heifers was analyzed by examining the distribution of each OTU across all samples. Figure 4.4 exhibits the average percentage of taxa shared by all samples at each level of taxonomy. This analysis revealed that 21% of the OTUs were present in all samples. A comparison of the unique OTUs associated with each of the diets was also completed (data not shown). During the acidotic challenge, 6% of OTUs were found to be unique to this period including *Atopobium* (2%), *Campylobacter* (2%), and *cc142* (2%). Uncharacterized genus *Wet75* was found exclusively in heifers fed forage (1%) while *RC1-13* was found to be a member (1%) of the core microbiome in heifers fed mixed forage. *Succiniclasticum* (3%) was only in the core microbiome of heifers fed high grain whereas during the recovery period no unique OTU's were associated with the core microbiome. At the family level, the average

Table 4.9. Correlation of pH variables to epithelial phylum. Only those phyla found to be significant are shown

		pH parameters								
Phyla		pH min	pH mean	pH max	Duration under pH 5.8 (min)	pH area under 5.8 (pH×min)	Duration under pH 5.5 (min)	pH area under 5.5 (pH×min)	Duration under pH 5.2 (min)	pH area under 5.2 (pH×min)
Actinobacteria	Correlation	-0.75	-0.60	-0.29	0.70	0.68	0.72	0.63	0.68	0.54
	P-value	<0.001	0.00	0.13	<0.001	<0.001	<0.001	<0.001	<0.001	0.00
Candidate division TM7	Correlation	0.53	0.57	-0.09	-0.54	-0.60	-0.60	-0.57	-0.56	-0.50
	P-value	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	0.01
Fusobacteria	Correlation	0.34	0.38	0.59	-0.25	-0.20	-0.17	-0.18	-0.15	-0.17
	P-value	0.07	0.04	0.00	0.19	0.30	0.39	0.34	0.44	0.38
Planctomycetes	Correlation	0.47	0.33	-0.06	-0.35	-0.25	-0.30	-0.20	-0.27	-0.16
	P-value	0.01	0.08	0.77	0.06	0.20	0.12	0.30	0.15	0.41
Tenericutes	Correlation	0.39	0.32	-0.01	-0.31	-0.30	-0.33	-0.28	-0.32	-0.23
	P-value	0.03	0.09	0.96	0.11	0.11	0.08	0.14	0.09	0.22

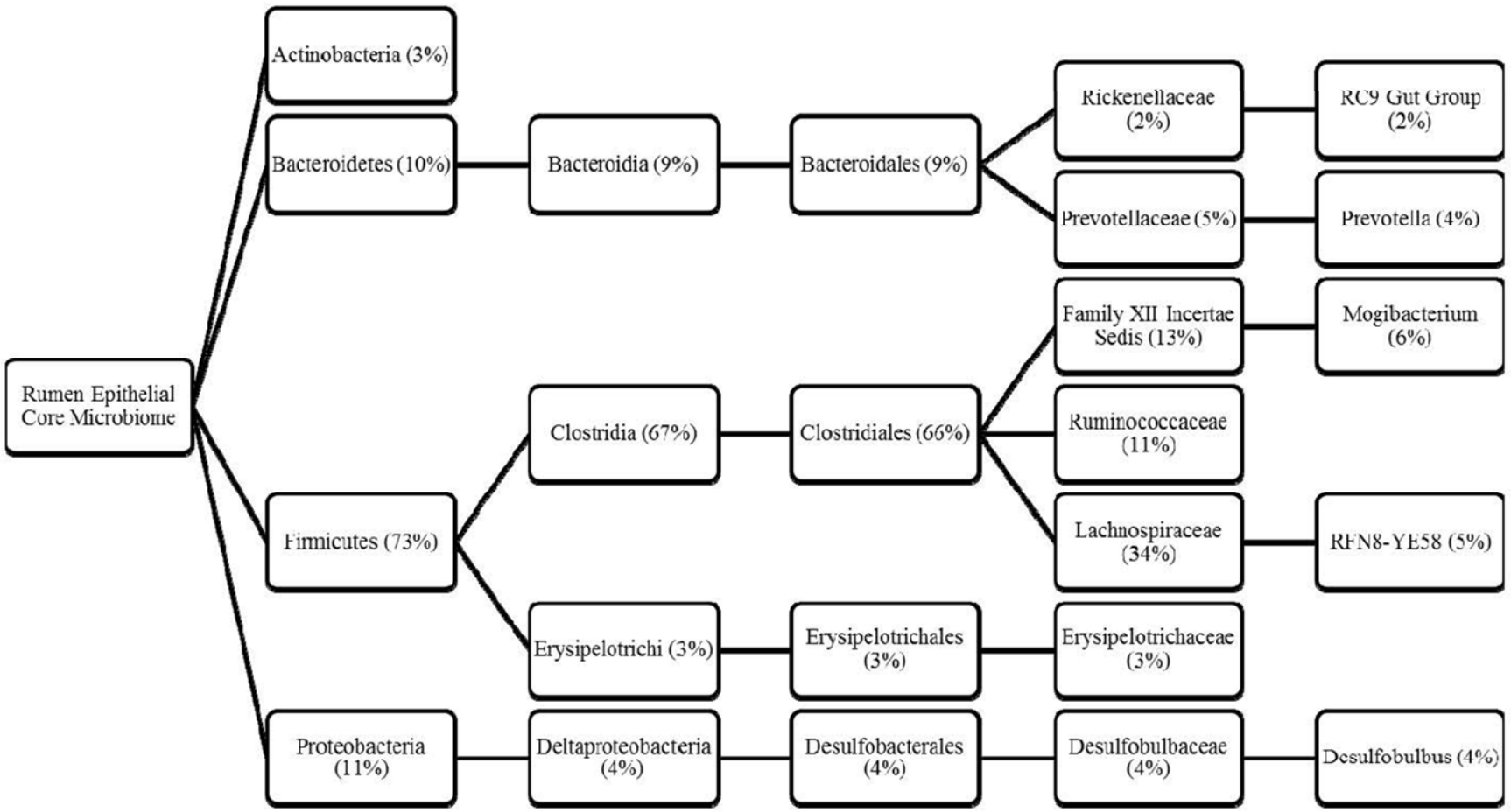


Figure 4.4. Graphical representation of the phylogenetic tree of the rumen epithelial core microbiome. Core microbiome was determined as any OTU present in all samples, for all heifers with all diets. Average percent abundance is shown in brackets.

percent abundance of OTU's with the highest representation included *Lachnospiraceae* (34%), *Family XIII Incertae Sedis* (13%), and *Ruminococcaceae* (11%), all members of the class *Clostridia* (67%).

4.3.6 Acidotic Challenge

The response of individual heifers to the acidotic challenge is shown in Table 4.10. Heifers 7 and 41 exhibited the lowest mean daily pH (4.00 and 3.93, respectively) and the highest area under pH 5.2. Whereas, heifers 41, 43, and 315 had the longest duration of time spent below pH 5.2 (Table 4.10). Based on the area under pH 5.2, heifers 7 and 41 were defined as subclinical acidotic and heifers 143 and 153 were defined as clinically acidotic (Figure 4.5). The increase in rumen pH in heifer 7 at 04:30 h reflected the administration of sodium bicarbonate as per animal care guidelines. *Lactobacillus* bacteria were 15% higher in heifers that exhibited clinical acidosis as compared to those that were subclinically acidotic (Fig. 5). Additionally, *Acetitomaculum, Megasphaera, Olsenella, RC39* and *Streptococcus* genera were increased by 2.5 – 4.5% in clinically acidotic animals. Taxa *E. nodatuim minitum, Comamonas, Desulfobulbus,* and *Sporobacterium* were all reduced by 1 – 4% in clinical as compared to subclinical acidotic heifers.

4.4 Discussion

The rumen microbiome associated with digesta is one of the most well studied microbial ecosystems (Krause and Russell, 1996). Ruminants depend on the rumen microbial community to convert otherwise indigestible feedstuffs into volatile fatty acids and microbial protein (Stewart *et al.* 1997). Interest in optimizing rumen function by manipulating the rumen

Table 4.10. Individual animal pH response to an Acidotic Challenge measured continuously over a 24 h period.

				pH<5.8		pH<5.5		pH<5.2	
Animal	Min	Mean	Max	Duration[1]	Area[2]	Duration	Area	Duration	Area
7	4.00	5.47	6.92	1224	1259	1107	913	857	619
41	3.93	4.31	7.15	1319	1896	1282	1505	1235	1127
43	4.75	5.21	6.87	1303	1020	1261	634	1015	284
143	5.12	6.04	6.96	666	242	413	85	92	2
153	5.05	6.01	6.83	893	431	761	178	259	12
156	4.81	5.86	7.09	935	432	615	208	348	56
315	4.68	5.33	7.07	655	555	631	361	1179	357
346	4.50	5.53	7.06	1186	830	969	514	730	248

[1] Duration of time spent below benchmark pH (5.8/5.5/5.2) measured in minutes

[2] Area under the benchmark pH (5.8/5.5/5.2) measured in pH x min

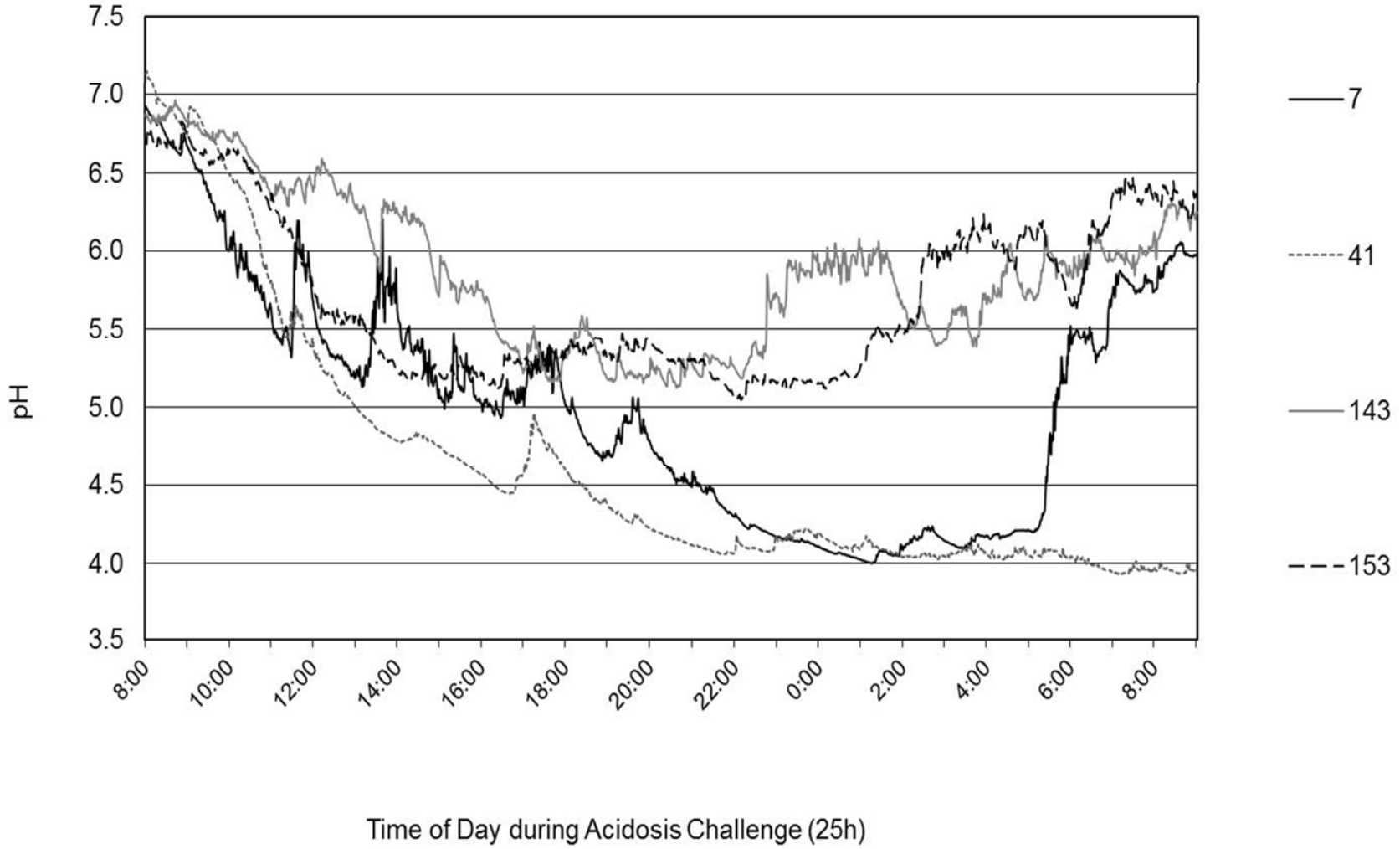


Figure 4.5. Daily measured pH in highly acidotic heifers (7 and 41) compared with heifers that were minimally acidotic (143 and 153). The time of the grain challenge was 09:00, and the time of feeding was 1 h post Challenge

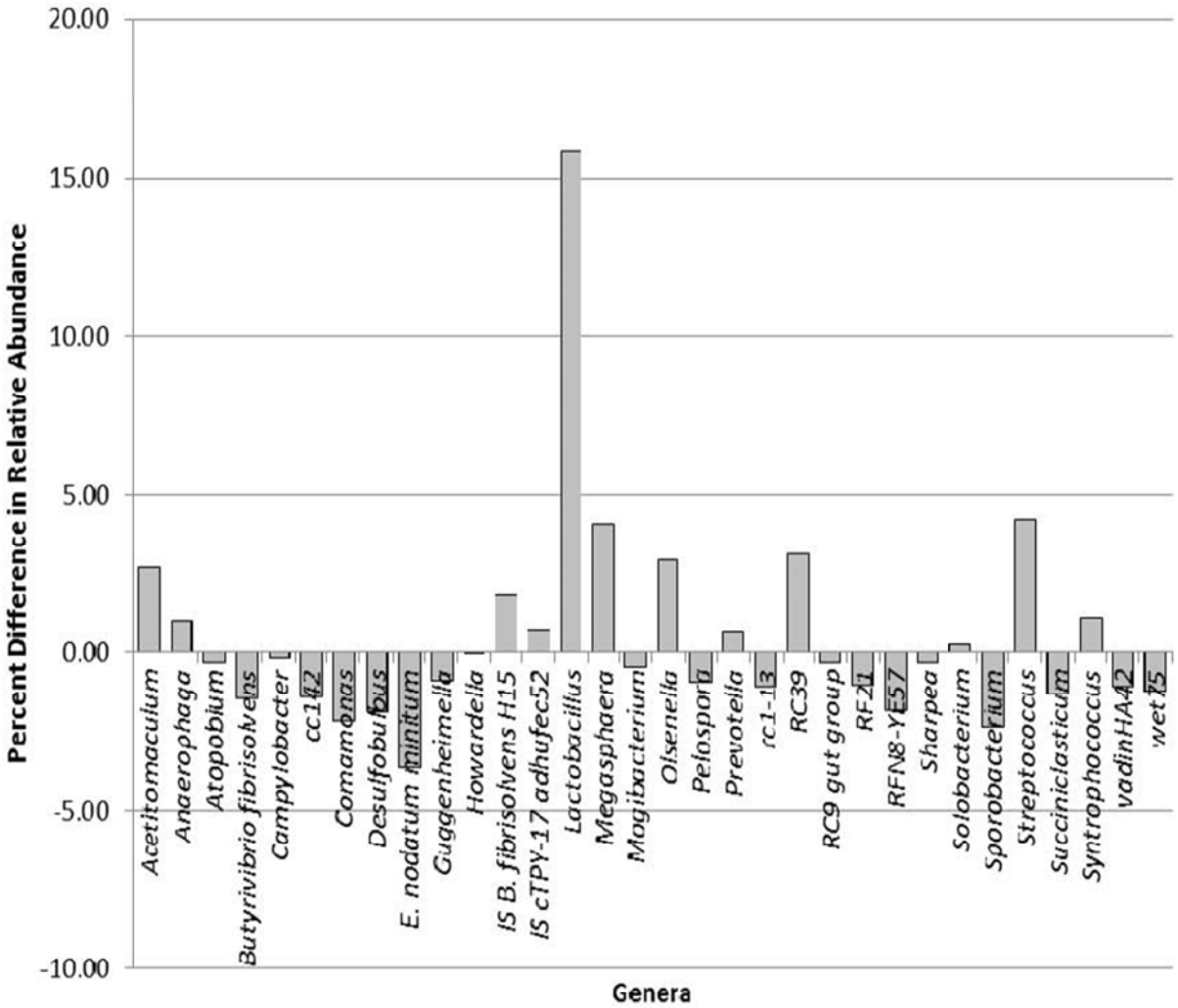


Figure 4.6. Difference in relative abundance (%) of epithelial bacteria genera in highly acidotic heifers (7 and 41) compared with heifers that were minimally acidotic (143 and 153). Acidosis ranking was determined by area under the curve for pH<5.2 adjusted for DMI.

ecosystem could be used to improve feed conversion, decrease methane emission, reduce nitrogen excretion, and prevent the shedding of pathogens (Stewart *et al.* 1997; Firkins *et al.* 2008). The aim of this study was to characterize bacterial epimural community in heifers fed forage, mixed forage-grain, high grain diets as well as during and after recovery from an acidotic challenge.

Original work described the adherent population of bacteria on the rumen epithelium as sparse and taxonomically heterogeneous (Cheng and Wallace 1979). The existence of a distinctive epimural population and the functions of these adherent bacterial populations in the rumen were first studied using the anaerobic roll-tube technique (Hungate 1969) in conjunction with light and electron microscopy (McCowan *et al.* 1978; Cheng *et al.* 1980; Rieu *et al.* 1990). These studies hypothesized that the rumen epithelial population had a role in oxygen-scavenging, tissue recycling, and the hydrolysis of urea. Phylogenetically, classical microbiology determined that this community contained a significant number of Gram-positive bacteria and that diet impacted the quantities of various species in the community population (Cheng *et al.* 1980). For example, *Bacteroides* spp. were found to predominant bacterial populations associated with the rumen epithelial in ruminants fed hay versus grain diets (Cheng *et al.* 1980). It was determined that the adherent microbial population consisted of a number of genera including *Micrococcus, Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Fusobacterium*, *Propionibacterium*, and *Selenomonas* as well as numerous unidentifiable bacteria (Cheng *et al.* 1980).

Within the past 10 years, the use of molecular biology has enabled more detailed classification of microbial phyla inhabiting the rumen. However, only a few studies have used these methods to analyze the rumen epithelial community. PCR based approaches including

cloning, DGGE and real-time PCR have been the primary techniques applied. However, depth and breadth of microbial analysis of the rumen has been limited by labour and equipment related costs impacting the number of clones, gels, primer sets and total number of samples that can be analyzed in a single experiment. Previous experiments employed cloning and found that *Firmicutes, Bacteroidetes*, and *Proteobacteria* were the predominant phyla on the rumen wall (Sadet-Bourgeteau *et al.* 2010; Chen *et al.* 2011). Of these, the *Firmicute* populations were most abundant in forage diets whereas in high grain diets the *Bacteroidetes* had the greatest abundance (Sadet-Bourgeteau *et al.* 2010). Characterization of the epimural community using PCR-DGGE (Sadet *et al.* 2007) has showed conflicting results with regards to the impact of dietary change on the diversity of rumen epithelial bacteria (Sadet *et al.* 2007; Sadet-Bourgeteau *et al.* 2010). Studies using a variety of molecular techniques have been able to show a strong epimural bacterial association to the individual in wethers, regardless of diet (Sadet-Bourgeteau *et al.* 2010) and increases in the total estimated population of rumen epimural bacteria corresponded to increases in molar proportions of acetate, isobutyrate, and isovalerate (Chen *et al.* 2011).

In the current study, three different molecular methods were used to determine the bacterial epimural community of heifers fed 5 different diets. These included PCR-DGGE, real-time PCR and next generation sequencing. While real-time PCR and DGGE are common techniques (Sadet *et al.* 2007; Chen *et al.* 2011), their application in the rumen is limited due to the large number of diverse species and, presence of PCR inhibitors and lack of sensitivity (DGGE) (Chen *et al.* 2011). However, despite these limitations there were a number of similarities between data obtained using real-time PCR and DGGE to that obtained from high through-put pyrosequencing. Of the 6 species/genera specific real-time PCR primer sets used, *Prevotella* spp.*, F. succinogenes, M. elsdenii* and *S. bovis* all produced treatment averages similar to what

was seen in the percent abundance data determined by the analysis of pyrosequencing data. Though the values were not exact, the trends in treatment differences were the same, indicating that real-time PCR is still a useful tool in identifying specific populations in a complex community. In this experiment, DGGE profiles were not found to differ significantly among diets; this was true in terms of both species diversity (Shannon and Simpson's indices) and richness (Chao1 and ACE). These results indicate that the bacterial community attached to the rumen wall of individual heifers was relatively stable in spite of a transition from a forage-through to a high grain diet. However, overall interpretation of these results in the rumen ecosystem is difficult due to a lack of comparative literature in the rumen of cattle. Similar results have been observed in sheep when the epimural community was examined under similar dietary regimes (Sadet *et al.* 2007). In the present study, treatment differences in sequencing data only became significant when comparing individual genera, and even then differences still tended to account for < 5% of the total bacteria. This adds further support to the idea that the epimural community is stable, with only slight shifts in bacterial communities with changes in diet. While DGGE cannot be used to accurately determine small genera/species level changes in the rumen epithelial populations, it can give an accurate overview of the epimural bacterial community. It is also important to note that due to large numbers of uncultured/unclassified species level taxa in sequencing databases and shorter sequence lengths, pyrosequencing information also has limitations in its ability to fully elucidate species level changes and therefore the full complexities of the rumen ecosystem.

Despite these limitations, recent advances in sequencing technologies have led to the wider use of metagenomic analysis for studying complex intestinal ecosystems such as the rumen (Deng *et al.* 2008). The popularity of using this approach has been based on the assumption that

genome sequences of abundant species will be well represented in a set of random shotgun reads, whereas species of lower abundance will have lower representation. In this approach, taxonomic classification is usually restricted to the level of genus as mismatches may occur as reference databases contain sequence from bacterial populations from the intestinal tract of humans, pigs, mammals and rodents. Reference databases will need to be enhanced to include more rumen microbial genomes as well as identify a greater number of unculturable species. The development of the "Hungate1000", a catalogue of 1000 reference microbial genomes from the rumen (http://www.hungate1000.org.nz/) should advance the relevance of high-throughput sequencing techniques to the rumen microbiome. Until such a database is fully developed, understanding of the rumen ecosystem requires amplified pyrotag deep sequencing analysis to provide a comprehensive assessment of an ecosystem's response to dietary change (Deng *et al.* 2008). Deep sequencing covering 98.8 to 99.0% of the rumen epimural community was able to provide a novel and detailed view of the impact of dietary change on this community. In addition, our reference database for alignment of sequences was developed specifically to highlight a number of rumen specific bacteria such as *Fibrobacter,* which are commonly underrepresented in reference databases. In this study, identification of unique OTUs required a collection of a minimum of 10 sequences with a 10% difference in sequence from any other OTU. Therefore, the lowest detection level of pyrosequencing data in this study required a minimum of 10 sequences out of the 9,323 sequences analyzed to indicate a single OTU. Based on this, 10 sequences accounts of 0.001% of the total rumen epithelial bacterial community sequenced, resulting in a very high detection level. Previously there have been relatively few bacterial species identified in studies of the rumen epithelial community. The use of pyrosequencing for the rumen epithelial bacterial community has only recently been applied to

pre-ruminant calves (Li *et al.* 2012b). Prior to this all sequencing of the rumen epithelial community was done using clone libraries (Cho *et al.* 2006; Sadet *et al.* 2007). Of these studies, only Chen *et al.* (2011) and Cho *et al.* (2006) were able to determine a number of previously undetermined bacterial taxa at the genera or species level based on the closest relative in the database. In these previous studies some of the novel taxa identified in the epimural community included *Desulfobulbus, Mogibacterium, Atopobium, Victivallis, Ottowia, Anaerovorax, Anaeroplasma* and *Synthrophococcus*. With pyrosequencing, Li *et al.* (2012) was able to additionally identify *Porphyromonas*, *Coprococcus*, *Acetivibrio*, *Sporobacter, Flavobacterium* and *Sedimentibacter*. While they studied calves up to 49 days of age, they identified many of the same taxa in the epimural community as found through cloning studies (Kocherginskaya 2001; Sadet *et al.* 2007) as well as a number of the taxa found in the current study with mature heifers. Due to the sensitivity of high-throughput sequencing, the present research identified 166 distinct species between the 5 dietary treatments, the majority of which have been previously unidentified in culture-based analysis of the rumen epithelial tissue and therefore could not be identified beyond the genus level. Some additional genera/taxa previously not described include *Adhufec269, Azonexus, cc142, Filifactor, Marvinbryantia, Sharpea, Solobacterium, Thermodesulfobium* and *Thermohalobacter*. The uncharacterized *Adhufec269* and *cc142* are not considered valid genera. However, both have been previously isolated from the hind gut of other mammals (Pryde *et al.* 1999). *Azonexus* is a part of the *Proteobacteria* phyla and the *Rhodocyclaceae* family, which are mainly denitrifying bacteria with versatile metabolic capabilities (Chen *et al.* 2011). *Filifactor* is a diverse genus within the class *Clostridia* which likely utilizes acetate and butyrate, but can potentially use a large number of other VFAs (Boone *et al.* 2001). *Thermosulfobacterium* and *Thermohalobacter* are both members of the class

Clostridia, a group of obligate anaerobes. While *Clostridia* taxa have been previously reported to be associated with the rumen epithelium (Cho *et al.* 2006; Chen *et al.* 2011), the abundance and diversity of these species is not fully understood. Previously, *Thermohalobacter* has been identified as a close relative of *Proteiniclasticum ruminis,* a strictly anaerobic proteolytic bacterium isolated from the rumen of a yak (Yang *et al.* 2010). *Sharpea* and *Solobacterium* from the order *Erysiopelotrichales* have only been recently established as a distinct class within the phyla *Firmicutes.* This class, order and family have been described as part of the gut microbiome in mammals (Morita *et al.* 2008) and were determined to be part of the core microbiome of the rumen epimural community in this study despite the fact that their metabolic role is unknown.

The concept of a core microbiome has been extensively researched in the human gastrointestinal tract, especially in relationship to a number of intestinal diseases including inflammatory bowel disease (Furrie 2006). This work in humans has relevance to intestinal microbial communities in other mammals especially as in humans the gut microbiota has been shown to play a key role in host health and energy metabolism (Furrie 2006). Similar to cattle, the human gut microbiome is dominated by four bacterial phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Harmsen *et al.* 2002). Research in humans has also found that each host has a unique biological relationship with its gut microbiota (Ley *et al.* 2006) that influences an individual's risk of disease (Furrie 2006). While the unique individual animal response to perturbations such as acidosis has long been understood in cattle (Schwartzkopf-Genswein *et al.* 2004; Bevans *et al.* 2005), the relationship between cattle and their gut microbiota, especially under abnormal conditions has not been fully elucidated. In humans, the key aim of the majority of gut microbiota research has been to understand if there are a number of essential species or strains that define a 'core microbiome' (DuPont and DuPont 2011). Such

work could then be used to define a "healthy state" with deviations from this core population being associated with disease (DuPont and DuPont 2011). However, the diversity of the human diet has made it difficult to define a core microbiome, as diet has a significant impact on the microbial composition of this intestinal community (Turnbaugh *et al.* 2006). In cattle, the herbivorous diet, while still diverse, is more limited in substrate scope as compared to the omnivorous diet of humans. Recently, the concept of a core microbiome was applied in lactating cattle (Jami and Mizrahi 2012) by pyrosequencing bacteria extracted from rumen digesta. However, the core microbiome of any mammal must be based on a diverse set of data including a variety of dietary regimes, in order to truly assess the necessity of the bacteria to the host and the ecosystem as a whole. Furthermore, analysis of the rumen content associated bacteria may not give an accurate overview of those bacteria that are most closely associated to the host, that being the population that is adherent to the epithelial tissue. Despite the continual sloughing of the stratified squamous epithelia and their complement of adherent bacteria (Cheng and Wallace 1979), the stability of the rumen epithelial tissue surface biofilm is potentially greater than that of digesta-associated biofilms because the enzymatic activities of this ecosystem are often integrated with those of the tissue itself. In the rumen, the members of the epimural community produce urease that the epithelial tissue is unable to produce but needs in order to convert urea to ammonia (Cheng and Wallace 1979). Due to close association, those bacteria firmly adherent to the rumen epithelial tissue would likely have the most impact on host health and arguably would be most appropriate bacteria for determining a 'core rumen microbiome'. While the thorough rinsing of the epithelium prior to sampling in this experiment ensured that samples taken represented only those bacteria which are adherent to the rumen epithelium and not a by-product of residual rumen fluid, it is important to recognize that in this dynamic ecosystem, and no

bacterial niche (i.e., solid, liquid or epithelial-adherent) is truly independent. Although a number of the dominant epimural bacterial detected at the class and family level taxa in this work are the same as those reported in rumen solid and liquid-associated bacterial populations, at the genus level many of the OTUs were not affiliated with rumen content-associated bacterial taxa and several genera were completely distinct (Stewart *et al.* 1997).

Diet is one of the major factors influencing the populations and metabolic function of the microbial community in the rumen (Tajima *et al.* 2001; Kocherginskaya 2001; Sadet *et al.* 2007). The nature of feed and the physicochemical changes induced by its fermentation are known to favor the development of certain microbial ecotypes in ruminal solid and liquid phases (Kim *et al.* 2011). Previous research in lambs showed that the epimural community was less influenced by diet than the microbiota associated with rumen contents (Sadet *et al.* 2007; Sadet-Bourgeteau *et al.* 2010). This stability may be due to the tissue specific metabolic activities of adherent populations and a reflection that this populations relies less on digesta to function (Sadet-Bourgeteau *et al.* 2010). However, so far experimental data have not been sufficient to describe in detail the changes in the rumen epithelial populations under conditions of severe pH change as occurs during subclinical and clinical acidosis.

The phyla most significantly impacted by diet and pH were not among the major three phyla based on total representation in the rumen adherent populations, but instead were *Acintobacteria, Candidate division TM7, Fusobacteria, Synergistetes* and *Tenericutes*. Additionally, all of these phyla except *Actinobacteria* decreased with low pH and were at highest abundance with forage, mixed forage or both diets. Correlation analysis indicates that these phyla are sensitive to low pH and those bacteria in the phyla *Candidate division TM7* were additionally sensitive to the amount of time that the rumen pH was 5.8 or lower. Members of the *Candidate Division TM7* have not

been previously reported to be associated with the rumen epithelium. Members of this phylum are classified as candidate division due to the lack of cultured representatives. Our lack of understanding of their metabolic function makes these taxa important for future research.

At the genus level, 61 key genera were identified, including two that were still phylogenetically labeled as unknown and unclassified. In the classical work performed by Cheng and Wallace (1980), all of the rumen epithelial genera belonging to the phyla *Actinobacteria* were different than what was identified in the current study. This classical work identified populations of *Micrococcus*, *Corynebacterium* and *Propionibacterium* whereas in the present study only *Atopobium* and *Olsenella* were identified. Members of the genus *Atopobium* and *Olsenella* are closely related and have only begun to be identified with the use of molecular microbiology in the epimural community of the rumen (Cho *et al.* 2006; Chen *et al.* 2011). Classically, these bacteria were considered unculturable and since they are Gram-positive, rod shaped bacteria similar to *Micrococcus, Corynebacterium,* and *Propionibacterium,* it is likely that they were misidentified in earlier rumen epithelial studies (Cheng *et al.* 1980).

An extensive amount of research has gone into identifying *Fusobacterium necrophorum* and its role in liver abscesses in cattle (Berg and Scanlan 1982; Tadepalli *et al.* 2009). *Fusobacterium* spp. were found in heifers fed the forage (1.1%) and mixed-forage (0.1%) diets, but not in the high grain diet. This is similar to previous studies that examined the rumen contents of cattle fed forage-based diets (Narayanan *et al.* 1997; Nagaraja and Titegemeyer 2007). Classical microbiology has shown that *Fusobacterium necrophorum* is a normal inhabitant of the rumen (Berg and Scanlan 1982; Tadepalli *et al.* 2009) and can be at least 10-fold higher in grain-fed cattle compared to forage-fed cattle ($>10^6$/g vs. $<10^5$/g of rumen contents) (Tadepalli *et al.* 2009). However, reports of *F. necrophorum* in the rumen have indicated that it can be found as a

free-floating organism or attached to feed particles and isolations of *Fusobacterium* from the rumen wall are limited and has only been associated with ruminal lesions (Berg and Scanlan 1982; Tadepalli *et al.* 2009). Pyrosequencing data from the present study confirmed previous findings that *Fusobacterium* are part of the commensal rumen epithelial community; however, they are not part of the core microbiome. Furthermore, in the present study it was found that *Fusobacterium* was more abundant in heifers fed forage, as they were not found to be associated with the epithelium under acidotic conditions, suggesting that they were sensitive to low pH. This sensitivity was confirmed by correlation analysis (Table 4.7) and supported by previous studies (Cho *et al.* 2006).

To our knowledge, no previous research has compared the long-term impact of an acidotic challenge on the rumen epithelial community. At the genus level, 9 different groups showed an increase or decrease during the recovery period as compared to the acidotic challenge. *Atopobium, Desulfocurvus, Fervidicola, Lactobacillus, Olsenella, Proteiniborus, RC39, Sharpea* and *Succinivibrio* were all more prevalent during the acidotic challenge. Other than *Sharpea* and *Succinivibrio*, all other genera returned to levels similar to those in the high grain treatment. The exact physiological significance of these two genera is unknown. However, the genus *Sharpea* from the order *Erysiopelotrichales* has only been recently established as a distinct class within the Firmicutes. This class, order and family have been described as part of the gut microbiome in horses, pigs, mice and humans (Kim *et al.* 2011) and have been identified as part of the core microbiome of the rumen epimural community in the present study. Members of the genus *Succinivibrio* from the phylum *Proteobacteria* and the class *Gammaproteobacteria,* have long been recognized as part of the rumen ecosystem, but has not been extensively studied due to low abundance within the rumen. However, future research into the significance of this bacterium in

the epimural community, especially in animals fed high grain diets, could help elucidate the role of this microbe and its negative correlation to mean daily rumen pH.

Individual heifers varied substantially in ruminal pH in response to an acidotic challenge (Figure 4.6) and this variability is also apparent during commercial beef production (Schwartzkopf-Genswein *et al.* 2004; Bevans *et al.* 2005). To date, analysis of rumen populations under acidotic conditions has been mainly limited to rumen contents and not to the epimural community (Khafipour *et al.* 2009). Under acidotic conditions, some bacteria are negatively impacted by the decreased pH associated with high lactate production whereas others such as *Lactobacillus*, *Megasphaera, Streptococcus, Succinivibrio*, and *Escherichia* increase with higher ruminal lactic acid concentrations (Nagaraja and Titgemeyer 2007; Khafipour *et al.* 2009). Research regarding *Lactobacillus* spp. within the rumen and specifically adherent to the epithelial wall is comparatively limited (Costerton *et al.* 1987) and the most recent publications using molecular based methods (Cho *et al.* 2006; Chen *et al.* 2011) have not been able to detect the presence of *Lactobacillus* on the rumen wall of cattle fed high grain diets. However, high levels of *Lactobacillus* were found adherent to the rumen epithelium of 6 out of the 8 animals during the acidotic challenge. This was not completely unexpected as *Lactobacillus* bacteria have long been associated with the commensal luminal and adherent populations in the gastrointestinal tracts of many mammals including humans (Heilig *et al.* 2002). However in this study, the presence of lactobacilli only in the acidotic challenge treatment and at levels 16% higher in heifers that were highly acidotic (7 and 41) compared to those that were minimally acidotic (143 and 153) was not anticipated. Furthermore, the highly acidotic heifers were also the only individuals with detectable *Streptococcus* populations, and both of these individuals experienced the most severe response (pH min $\leq$ 4.0) to the acidotic challenge (Figure 4.6).

Table 4.11. Rumen fermentation variables measured in heifers during transition from Forage, Mixed Forage, High Grain, Acidotic Challenge and Challenge Recovery diets*.

Rumen Fermentation Variable	Treatment					SEM	*P*-value
	Forage**	Mixed Forage	High Grain	Acidosis Challenge	Challenge Recovery		
Mean nadir		5.78b	4.96a	4.61a	5.08a	0.12	<0.001
Mean daily pH		6.36b	5.99b	5.47a	6.09b	0.12	<0.001
Mean maximum pH		6.95b	6.62a	7.00b	6.56a	0.07	<0.001
Rumen pH≤5.8							
Duration (min day^{-1})		115c	828ab	1023a	512b	103	<0.001
Area under (pH ×		25c	448ab	833a	186bc	106	<0.001
Rumen pH≤5.5							
Duration (min day^{-1})		25b	616a	879a	232b	88	<0.001
Area under (pH ×		4b	229ab	550a	77b	85	<0.001
Rumen pH≤5.2							
Duration (min day^{-1})		0c	414ab	715a	101bc	99	<0.001
Area under (pH ×		0b	78ab	338a	29b	68	<0.001
Total VFA, m*M*	76.8a	91.8ab	144.2c	128.1abc	129.5bc	5.8	0.002
Acetate (A), mmol/100mol	67.8b	61.8b	46.6a	51.5a	50.6a	1.4	<0.001
Propionate (P), mmol/100mol	17.8a	19.7a	37.5b	32.3b	33.1b	1.5	<0.001
Butyrate, mmol/100mol	3.84b	3.27b	1.27a	1.69a	1.84a	0.17	<0.001
Lactic acid, m*M* ***			0.71a	2.96b	0.03a	0.41	0.02

*Letters in each row indicate significant difference between treatments. The pH variables are a mean value for all heifers within a dietary treatment for the 24 h period starting at 08:00 h on the day of bacterial sample collection. The VFA and lactic acid concentrations are mean values for all heifers on a dietary treatment for samples taken 4 h post-challenge.

** pH values were unavailable during the Forage treatment

*** Lactic acid values were undetectable for Forage and Mixed Forage treatment.

This extreme acidotic environment was indicative of an acute acidosis compared to the other heifers in this study as measured by pH minimum and duration of pH under 5.2 (Table 4.11). *Streptococcus* spp. have long been understood to be a key bacteria in the "acidosis spiral" theory (Russell and Hino 1985), which states that *Streptococcus* is a commensal bacterium which persists at low levels in forage diets due to limited substrate availability. In ruminants fed increasing levels of starch, *Streptococcus* spp. produce lactate, decreasing ruminal pH and negatively impacting the growth of other bacterial species (Nagaraja and Titgemeyer 2007). In this study, lactic acid levels were numerically higher in heifers that experienced the severe challenge (7 and 41) corresponding with a 4% increase in *Streptococcus* spp. compared to heifers 143 and 153 which were subclinically acidotic (Table 4.12). The presence of *Lactobacillus* and *Streptococcus* at increased levels in heifers 7 and 41 indicates a clear correlation between the growth of *Lactobacillus*, *Streptococcus* and an increased severity of response to the acidotic challenge in the host. Whether these two bacterial groups are causative agents or the product of the acidotic conditions is unclear from the data. The proliferation of these same groups has been found to occur in the rumen digesta fraction (Tajima 2000; Khafipour *et al.* 2009). However, the presence of *Streptococcus* is more transient and has mainly been found in ruminants that have not been previously adapted to a high grain diet (Nagaraja and Titgemeyer 2007). Furthermore, neither classical nor molecular methods have been able to clearly and consistently show the correlation between *Streptococcus* and acidosis in either the solid, liquid or epithelial fractions of the rumen (Goad *et al.* 1998; Tajima 2000; Chen *et al.* 2011). The lack of a consistent bacterial response across rumen studies might be due to the environmental conditions associated with each feeding regime.

Table 4.12. Rumen fermentation parameters including pH, volatile fatty acids and lactic acid averaged in individual heifers during transition from Forage, Mixed Forage, High Grain, Acidotic Challenge and Challenge Recovery diets.*

	Individual Heifer									
Fermentation variables	7	41	43	143	153	156	315	346	SEM	*P*-value
Mean minimum pH	4.79	5.23	4.90	5.21	5.46	5.08	5.09	5.08	0.10	0.26
Mean daily pH	5.84	5.88	5.65	6.14	6.25	5.98	5.96	6.13	0.08	0.31
Rumen pH≤5.8										
Duration (min day^{-1})	788	494	1002	427	480	651	535	581	78	0.06
Area under (pH x min)	509	557	625	131	167	319	319	356	77	0.27
Rumen pH≤5.5										
Duration (min day^{-1})	554	455	787	195	272	408	391	446	75	0.05
Area under (pH x min)	311	415	348	43	53	164	180	206	57	0.38
Rumen pH≤5.2										
Duration (min day^{-1})	342	372	577	57	65	274	454	320	69	0.09
Area under (pH x min)	178	290	141	7	3	61	122	88	41	0.51
Total VFA, m*M*	127.2	119.8	121.2	136.2	107.6	136.5	137.1	107.9	5.8	0.43
Acetate (A), mmol/100mol	50.4a	57.1b	53.9ab	50.8a	53.9ab	51.2ab	50.9a	53.3ab	1.4	0.01
Propionate (P), mmol/100mol	32.5	28.2	31.3	33.1	26.1	31.8	33.4	30.3	1.5	0.18
A:P (acetate: propionate)	1.65	2.43	2.07	1.57	2.39	2.06	1.84	2.03	0.17	0.17
Butyrate, mmol/100mol	10.92	9.54	10.82	11.11	13.49	11.43	10.54	10.83	0.47	0.78
Lactic acid, m*M*	1.00	3.02	0.36	0.22	0.61	0.16	0.25	2.57	0.41	0.27

*Letters in each row indicate significant difference between treatments. The pH variables are a mean values for all heifers by dietary treatment for the 24 h period starting at 08:00 h on the day of bacterial sample collection. The VFA and lactic acid concentrations are mean values for all heifers on a dietary treatment for samples taken 4 h post-challenge.

Potentially, the stability of the rumen epithelial community can only be truly disrupted by an extreme change in environmental conditions, such as an acidotic challenge, supporting the hypothesis that this community is normally highly stable.

It has been previously observed that some of the variability in host response to low pH is related to variation in VFA absorption (Penner *et al.* 2009). Under extreme acidosis conditions, the buffering capacity of many animals is overloaded by the high levels of VFAs produced in a short period of time. However, those animals that exhibit higher VFA absorption on the apical surface of rumen epithelial cells have a greater ability to mediate the effects of increased VFA production than those animals, which have less uptake capacity (Penner *et al.* 2009). However, uptake capacity does not fully explain individual animal variability in susceptibility to acidosis and potentially to variation in the metabolism of the epimural community may also contribute to this variability.

4.5 Conclusions

Though we were unable to ascertain the metabolic properties of the epimural community from this study, further use of metagenomic and metatranscriptomic technologies could potentially reveal the source of animal variability in susceptibility to acidosis. Molecular techniques have shown that the rumen microbial community is far more complex than originally believed based on traditional culture techniques. This study represents the largest bovine epimural pyrosequencing effort to date in terms of both the number of individual samples and the depth of sequencing (average 4267 reads per sample). The added value of characterizing low-abundance community members is clearly illustrated in the increased ability to detect rumen bacteria affected by dietary treatment. Previous research has indicated that individual animal variability has hindered an accurate description of the rumen microbiome by masking the effects

of treatment on these populations (Chen *et al.* 2011; Petri *et al.* 2012) Yet this study showed dietary treatment variation (sequence and qPCR bacterial enumeration) and animal variation (DGGE) even when using diets similar to those previous studies (Petri *et al.* 2012). This indicates that the depth of sequencing was such that it removed the effect of animal as a masking variable for treatment effects. Furthermore, many of the bacterial genera impacted by dietary change were found to account for less than 5% of the total epimural population. These low-abundance organisms may be extremely active and have major impacts on the overall rumen environment. Because detail regarding the effects of dietary treatment and the variation between animals was seen here predominantly at the genera level, the data suggests that it is the less abundant and possibly rare community members impacting rumen fermentation changes. Based on these findings, it is evident that the continued application of deep-sequencing approaches will promote the discovery of less-abundant and rare community members and will help to provide a better understanding of the importance of these microorganisms in digestive disturbances such as acidosis. While culture independent genomic techniques are not without limitations, as they are unable to infer bacterial function, and it is important to note that genomic based research will only be able to describe the potential for a healthy or disease state based on the microbial community populations.

Chapter 5 describes the analysis of the rumen liquid and solid microbial composition using the same three molecular methods used in the previous chapter. This chapter meets the objectives of identifying changes and determining inherent variability in the microbial populations in response to diets which may predispose feedlot cattle to acidosis.

CHAPTER 5
5.0 CHARACTERIZATION OF DIGESTA AND FLUID ASSOCIATED RUMEN BACTERIAL MICROBIOME IN INDIVIDUAL CATTLE

5.1 Introduction

The rumen microbiome is an extremely diverse and well-studied microbial ecosystem (Hungate 1966; Latham *et al.* 1971; Russell and Hespell 1981; Krause and Russell 1996). Rumen acidosis is among the most researched rumen conditions because of its negative impact on cattle production, reducing feed intake and decreasing productivity (Nagaraja and Lechtenberg 2007; Nagaraja and Titegemeyer 2007). Ruminal acidosis occurs when cattle consume readily fermentable substrates such as grain resulting in an increase the concentrations of acid in the rumen leading to, a drop in ruminal pH (Nagaraja and Lechtenberg 2007). The role of rumen bacteria in the rapid conversion of grains to organic compounds, such as lactic acid, has been predominantly studied using traditional culture techniques to identify the rumen's microbial inhabitants, their preferred substrates and the products of substrate fermentation (Hungate 1966; Cheng and McAllister 1997; Flint 1997). However, the development of molecular techniques to investigate ecological microbial communities has indicated that the traditional culture techniques have underestimated rumen bacterial diversity (White *et al.* 1999; Yu and Morrison 2004; Sadet *et al.* 2007; Dowd *et al.* 2008b). Metagenomics has been defined as the science of biological diversity; combining the use of molecular biology and genetics to identify and characterize genetic material from complex microbial environments, from multiple individuals and with significantly less labor than other molecular methods such as DGGE, real-time PCR and cloning (Deng *et al.* 2008; Kinross *et al.* 2011; Jami and Mizrahi 2012; Luo *et al.* 2012). Extensive use of next generation sequencing methods in exploration of the human gut microbiome has shown that

phylotype composition can be specific and stable in an individual (Costello *et al.* 2009) and that an individual conserves over 60% of phylotypes of the gut microbiome over time (Manichanh *et al.* 2008). This implies that each host has a unique biological relationship with its gut microbiota (Sartor 2004; Ley *et al.* 2006a), and this relationship can influence an individual's risk of disease (Kinross *et al.* 2011). Though the gut microbiome varies between species, many of the techniques and theories about the human gut ecosystem likely apply to other mammals including cattle, despite inherent physiological differences among hosts. In human research, there has been a significant effort to define a 'core' microbiome to establish a baseline for a healthy gut and from that point identify deviations in the gut microbiome that are indicative of disease (Turnbaugh *et al.* 2006, 2007). Initial metagenomic studies examining the changes in ruminal bacterial communities during the feeding cycle (Jami and Mizrahi 2012), and in the epimural community across feeding regimes (Chapter 4) have indicated that similar to humans, a number of bacterial taxa are consistently identified despite the variation in the rumen bacterial community between individual hosts (Chen *et al.* 2011; Jami and Mizrahi 2012).

Given that bacteria within the rumen have proliferated based on their specialized ability to degrade substrates, it is clear that changing the type and availability of dietary components would have the greatest impact on the composition of the rumen core microbiome (Jouany 1991). Classical microbiology has shown that the largest impact on the rumen microbiome through dietary manipulation is through the induction of clinical acidosis (Russell and Hino 1985). Therefore, the objectives of this experiment were to examine populations during transition from forage to concentrate diets, and then during an acidotic challenge, an approach most likely to cause a dramatic change in the core microbiome.

5.2 Materials and Methods

5.2.1 Animals and sampling

This study derived data from an experiment that investigated the impact of an acidotic challenge on rumen function (Schwaiger *et al.* unpublished). The present study used eight ruminally canulated Angus heifers from the original 16 animals. These eight were selected based on the fact that these individuals were adapted to diets for a longer duration to allow the bacterial community to stabilize prior to sampling on each treatment (Chapter 4). Heifers were cared for in accordance with the guidelines of the Canadian Council on Animal Care (Olfert *et al.* 1993). Briefly, having never been fed grain prior to this experiment, heifers (BW: 308kg ± 35 SD) were assigned to one of four blocks, based on equal starting body weights for all blocks and then received a progression of 5 dietary treatments over 11 wk. Heifers were fed grass-hay (forage) with a mineral supplement (Chapter 4) for a minimum of 3 wk prior to sampling (d -1). They were then transitioned using a single step to a mixed forage – concentrate (mixed forage) diet consisting of 60% barley silage, 30% barley grain and 10% supplement (dry matter basis) and remained on the mixed forage diet for 2 wk prior to the second sampling. After sampling, heifers were transitioned over 20 d to a high grain diet (high grain) consisting of 81% barley grain, 9% barley silage and 10% supplement (dry matter basis). They remained on the high grain diet for 34 d to allow the rumen microbiome to stabilize before collection of the third sample (d 69). One week later the heifers were subjected to an acidotic challenge on d 76. As previously reported in Chapter 4, the challenge model involved restricting intake to 50% of the average ad libitum as-fed intake, based on a percentage of body weight for each heifer. Average intake was determined for each heifer using the individual feed intake (as-fed) 31 d prior to the challenge, and weekly thereafter. . After 24 h of feed restriction, a single dose of ground dry-rolled barley

grain ground through a 4.5 mm screen was introduced directly through the rumen cannula. In an effort to simulate acidosis, heifers in replicate 1 received a dose of 20% I: BW (as-fed). However, as this challenge was found to be too severe based on low ruminal pH, the remaining heifers received a dose of 10% I: BW (as-fed). Beginning at the time of challenge, then every 2 h for the first 12 h and then every 4 h for the next 12 h, rumen pH was monitored using strained ruminal fluid from the ventral sac and a portable pH meter (Accumet 25, Fisher Scientific). If ruminal pH was below 4.2, an additional pH measurement was made 1 h later. If ruminal pH remained ≤ 4.2, heifers were dosed with 250 g of sodium bicarbonate. Rumen content samples were also collected 1 wk post-challenge (challenge recovery) to monitor the degree of recovery after an acidotic challenge while heifers continued to receive the high-grain diet. Composition of the diets can be found in Chapter 4, Table 4.1 (Schwaiger *et al.* unpublished).

5.2.2 Rumen sampling

Sampling of rumen contents for bacterial analysis occurred at 4 h post-feeding on the collection day for each dietary treatment, except on the day of acidotic challenge when an additional sample was collected at 12 h post-feeding. Rumen bacteria samples were collected via a rumen cannula with a wide mouth 500 mL Nalgene centrifuge bottle (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) from multiple sites (cranial, caudal, dorsal, caudal ventral) within the rumen and the bottle was immediately sealed to retain a near anaerobic environment.

A subsample of rumen contents was also taken at 4 h and 12 h post feeding to measure volatile fatty acid (VFA) and lactic acid concentrations for each treatment period. In-dwelling pH was recorded on the day of sample collection (every minute) starting at 0800 h. A more extensive study of the diurnal pattern of VFA concentrations and ruminal pH is presented by Schwaiger *et al.* (unpublished). The pH was measured using the Lethbridge Research Center

Ruminal pH Measurement System (LRCpH; Dascor, Escondido, CA; Penner *et al.* 2006). The daily ruminal pH data were summarized as minimum pH, mean pH, maximum pH as well as duration and area under the curve below the benchmarks of pH 5.8, 5.5 and 5.2 (Penner *et al.* 2006) These data have been previously reported in (Chapter 4).

5.2.3 Bacterial DNA extraction and pyrosequencing

Samples were processed immediately upon collection as per the methodology of Wang *et al.* (2011). Briefly, liquid-rumen samples were obtained by mixing the collected rumen contents in the centrifuge bottle, placing those contents into a heavy walled 250 mL beaker and separating the particulate and liquid using a Bodum coffee filter plunger (Bodum Inc., Triengen, Switzerland). Fluid-digesta aliquots of 5 mL were place in aluminum foil dishes and flash-frozen in liquid nitrogen and stored at -80°C. Solid rumen content samples were collected by removing the remainder of the liquid contents with the Bodum coffee filter and then flash freezing subsamples (~5g) in liquid nitrogen and storing at -80°C until further processing.

Genomic DNA was extracted as described by Kong *et al.* (2010). Samples were first manually ground to a fine powder in liquid nitrogen using a mortar and pestle, and then combined with proteinase K (1mg/mL; Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) and further ground in liquid nitrogen using a Retsch RM100 grinder (Retsch GmbH, Haan, Germany). Samples were processed individually with sterilization of the grinder surface (12% aqueous sodium hypochlorite in water for 15 min, followed by 15 min of exposure to UV) between each sample. Samples of liquid and solid- digesta were processed on separate days to avoid cross contamination. Each sample was mixed with ~100 mL of liquid nitrogen and transferred to a 200 mL wide-mouth centrifuge bottle and incubated for 40 min at 50°C in a water bath to thaw the samples. After incubation, 15 mL of sample was transferred into a 50 mL

polycarbonate tube (SS34; Fischer Scientific Ltd, Nepean, Ontario, Canada) containing 1.5 mL of 20% vol/vol SDS (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada). The resultant mixture was then incubated for 45 min at 65°C in a water bath. After incubation, samples were centrifuged at 10,000 × g for 10 min and three equal volumes of supernatant were combined with a preheated (65°C) 2% agarose mixture (Sigma-Aldrich Canada Ltd., Oakville, Ontario). The suspension was gently inverted to create a homogenous mixture and transferred to petri dishes (15 mm H; Fischer Scientific Ltd, Nepean, Ontario, Canada) and allowed to cool at room temperature. Once set (1 h), agarose samples were cut into strips (1 cm wide) and washed in 10 volumes of TE buffer (10:2 of 1M Tris-HCl to 0.5M EDTA) for 16 h. Agar (200 mg) containing cleaned sample DNA were distributed between triplicate 1.5 mL snap cap tubes (Fischer Scientific Ltd, Nepean, Ontario, Canada) and placed in -80°C for 1 h. Frozen samples were "freeze-squeezed" (Thuring, 1975) by centrifuging at 10,000 × g for 10 min to extract the DNA fragments from the agar. The resulting supernatant of TE buffer containing bacterial DNA was transferred to a new 1.5 mL tube. Samples were then refrozen at -80°C for 1 h and centrifuged once again. Supernatants were combined from the repeat centrifugation and all samples were stored at 4°C prior to analysis. The DNA from each sample was quantified using fluorometric dsDNA with picogreen dye (Invitrogen, Life Technologies Inc., Burlington, ON, Canada) and measured with a synergy HT plate reader (BioTek U.S. Ltd, Winooski, VT, United States). Subsequently, individual genomic DNA samples for all treatments were diluted to a concentration of 20 ng μL^{-1} in TE buffer. One 20 µL aliquot of each sample for a total of 36 genomic DNA samples (forage n=5; mixed n=8; high grain n=7; acidotic challenge n=8; challenge recovery n=8) were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for pyrosequencing using a 454 GS FLX Titanium Sequencing System (454 Life Sciences, a

Roche company, Branford, CT, USA). Pyrosequencing targeted the V1 to V3 hypervariable region of the 16S rRNA gene as described by Dowd *et al.* (2006).

5.2.4 PCR-DGGE analysis

Extracted, diluted DNA (3 µL of 20 ng μL^{-1}) from each sample was added as template to amplify the V3 region of the 16S rRNA gene for PCR-DGGE analysis in a 25 µl reaction. Amplification was performed using Qiagen HotStar Plus Master Mix Kit (Qiagen) and 500 nM of forward and reverse primers (341f with GC-Clamp:CGCCCGCCGCGC-GCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG and 534r: ATTACCGCGGCTGCTGG) developed by Muyzer *et al.* (1993) as previously reported (Petri *et al.* 2012). Polymerase chain reaction conditions were 95°C for 5 min, 94°C for 30 s, temperature gradient decreasing from 65°C to 55°C by 0.5°C each cycle for 30 s, 72°C for 1 min for 20 cycles, followed by 94°C for 30 s, 56°C for 30 s, 72°C for 1 min for 10 cycles and 72°C for 10 min for final elongation. Amplified DNA was assessed for quality using gel electrophoresis and quantified using flurospectrophotometry by measuring the $A_{260/280}$ (ND-3300 Nanodrop, Wilmington, DE, U.S.A). Amplified DNA was then normalized to 100 ng μL^{-1} and 4 µL DNA along with 4 µL of 2 x loaded dye (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol w/v in H_2O, pH 8.0) were placed in each lane on 8 % acrylamide gels with a 45 – 60 % denaturing gradient of urea and formamide. Electrophoresis was performed at 60 °C and 40 V for 20 h. Three lanes on each gel were loaded with DDGE Marker II (Wako, Nippon Gene, Japan) to provide both an internal and external marker. Gels were stained with SybrGold Nucleic Acid Gel Stain (Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A) according to manufacturer's instructions and photographed by UV transillumination.

5.2.5 Real-time PCR

As previously reported in Chapters 3 and 4, quantitative analysis was performed to quantify the relative abundance of 16S rRNA genes of seven bacterial species as a percentage of total eubacterial 16S rRNA, using the primers previously reported (Chapter 3.3.3). No deviations were made from the methodology reported there.

5.2.6 Pyrosequence analysis

Pyrosequencing analysis of the V1-V3 region of 16S rRNA on 93 samples yielded 613,689 raw reads. Reads with an average quality score of less than 35, as well as homopolymers greater than eight bases, and sequences with one or more ambiguous bases were removed from the sequence set. Sequences were then aligned against the SILVA database for 16S rRNA genes to define operational taxonomic units (OTUs; Schloss *et al.* 2009). Sequences that did not overlap the alignment region were also removed from the dataset. Sequences were trimmed so that reads overlapped in the same alignment space (Schloss *et al.* 2009) resulting in read lengths ranging from 167 to 349 bps. Pyrosequencing noise due to base call errors were minimized in the dataset using the pre-cluster algorithm in MOTHUR (Huse *et al.* 2010), whereby rare sequences highly similar to abundant sequences were re-classified as their abundant homologue. Chimeras were removed from the samples using the sequence collection (UCHIME) as its own reference database (Edgar *et al.* 2011). A distance matrix was constructed using the average neighbor algorithm at 0.03 (equivalent to species), 0.05 (genus), 0.25 (phylum) phylogenetic distances. Pairwise distances between aligned sequences were calculated at a 0.97% similarity cutoff and then clustered into unique OTUs. In total, there were 407,373 high quality reads with an average of 4,367±409 reads and 128±9 unique OTUs for each sample from each individual heifer

MOTHUR was also used to calculate the coverage (rarefaction curves; Figure 1), the number of species represented in a sample (species richness) using Chao1 and abundance-based coverage estimation (ACE), as well as the number of equally-abundant species (species diversity) with Shannon-Weiner and Simpsons indices (Table 5.1), and to create a dendrogram (Figure 5.2) based on treatment differences using the Jaccard index (Schloss *et al.* 2009). Estimation of the percentage of sequences within taxonomic classifications at the genus and species level was performed using a custom summation script.

5.2.7 Statistical analysis

Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 5.1, Applied Maths, Inc., Austin, TX, U.S.A) to create similarity matrices to identify community population differences among treatments and individual animals. Bands were visually selected based on peak height. Using average Dice's similarity coefficient (D_{sc}) index, with an optimization of 1.0% and with a tolerance of 1.0%, clustering was carried out using the unweighted pair group method with arithmetic means (UPGMA). Read number, sample coverage, unique OTUs, sample richness (Chao1 and ACE) and sample diversity (Shannon-Weiner and Simpson's indices) were compared with one-way ANOVA using the Proc Mixed

Table 5.1. Summary of average individual animal comparisons for unique OTUs, richness estimates, and diversity indices within rumen digesta. The minimum number of unique OTUs in each population was determined with a 10 % difference level.

	Individual Animals									
	7	41	43	143	153	156	315	346	SEM	*P*-value
Number of Sequences	4450abc	5521c	5243bc	3698abc	3868abc	3296a	3436ab	5427c	409	<0.001
Coverage (%)	91.9a	99.2b	99.1b	91.9a	94.9a	93.0a	94.2a	99.0b	1.8	0.01
Total # of Unique OTUs	107abc	152d	151d	130abcd	141bcd	94a	103ab	147cd	9	<0.001
Richness Estimate										
Chao1	146abc	195c	198c	175abc	184bc	124a	134ab	197c	12	<0.001
ACE	163ab	196b	200b	180ab	195b	131a	138a	204b	12	<0.001
Diversity Indices										
Shannon-Weiner	2.68ab	3.03bc	2.83abc	3.00bc	3.20c	2.76abc	2.50a	2.93abc	0.10	<0.001
Simpson's	0.16ab	0.14ab	0.18b	0.10a	0.10a	0.15ab	0.20b	0.16ab	0.02	<0.001

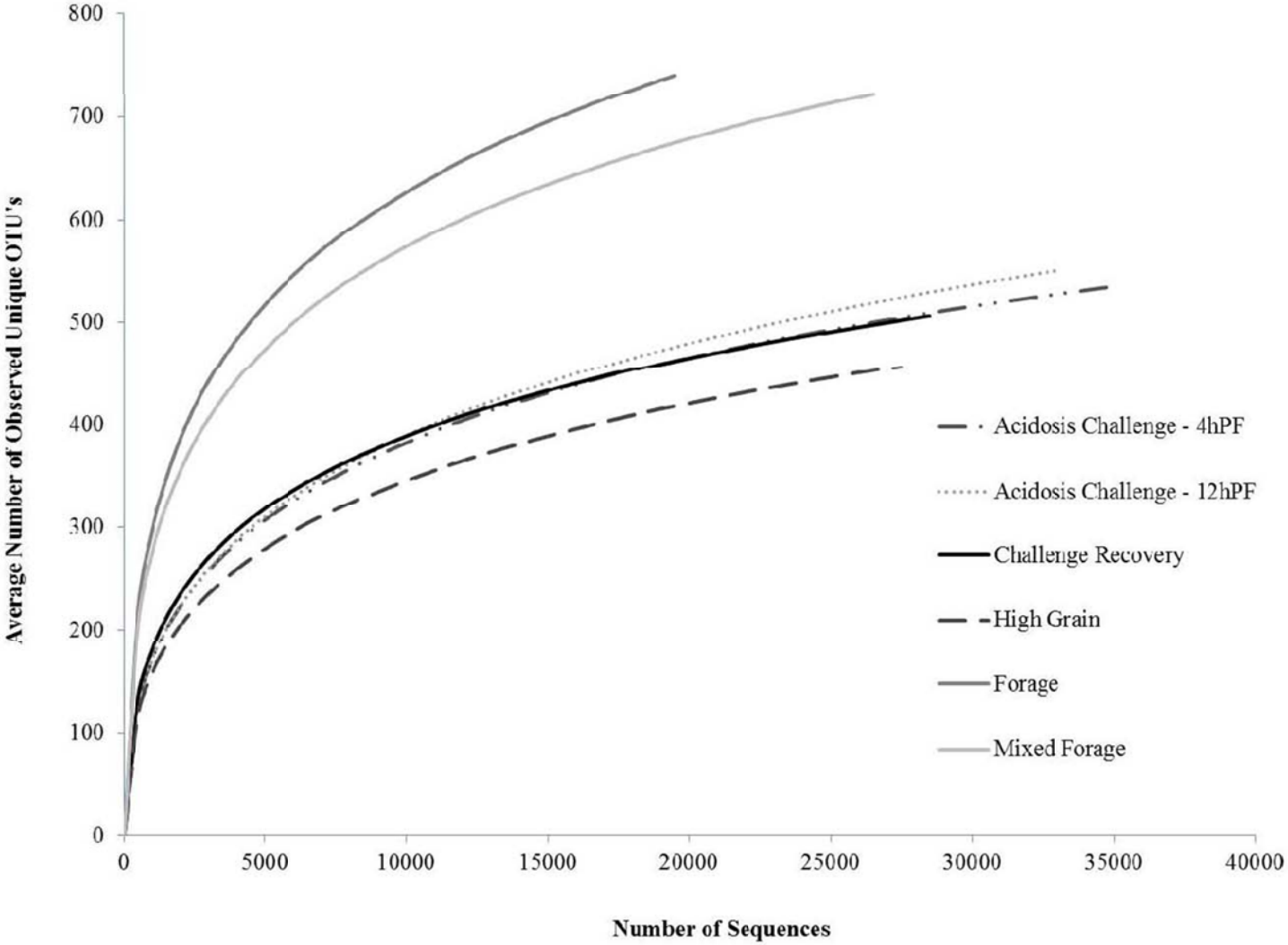
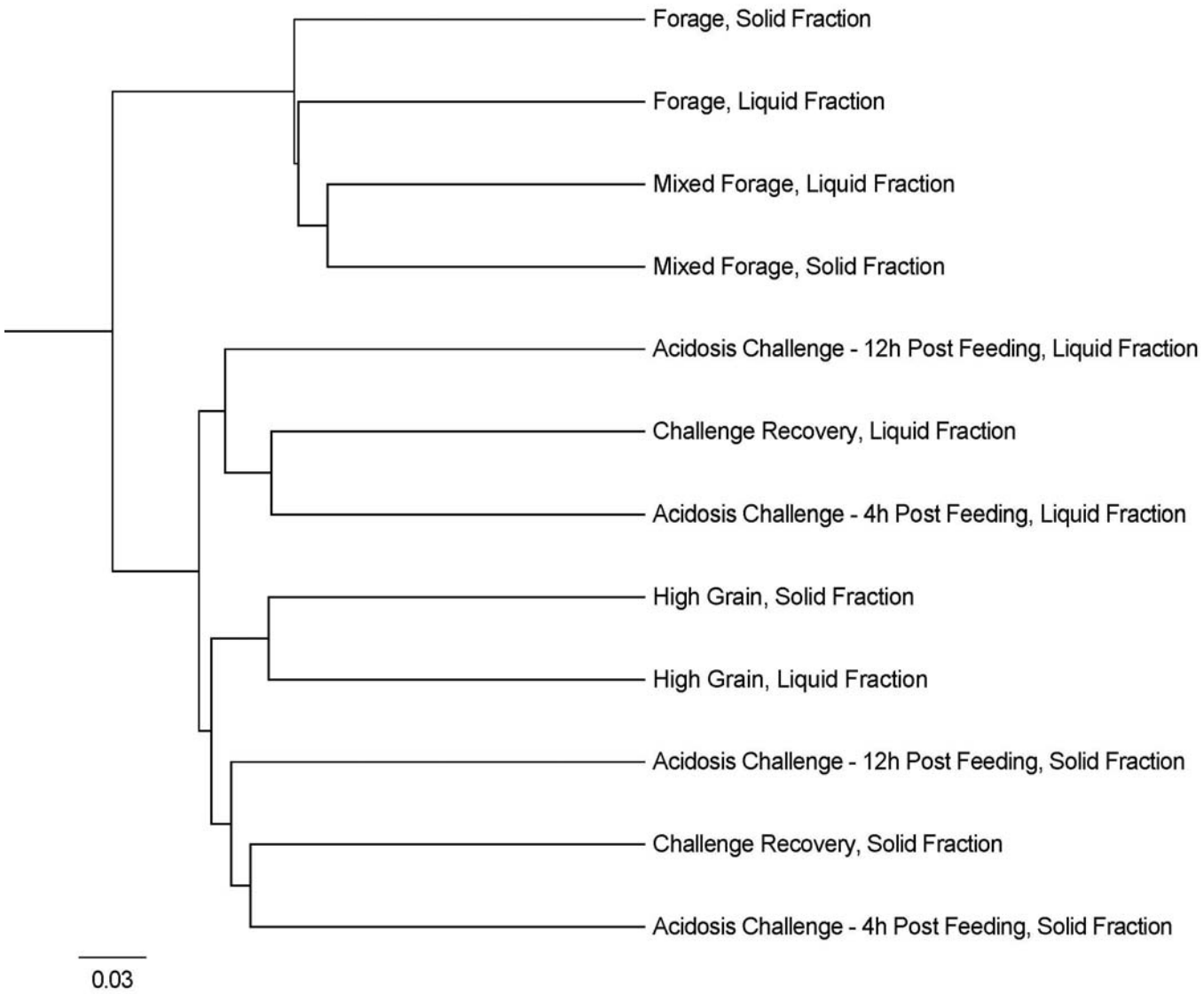


Figure 5.1. Rarefaction curves for rumen bacterial communities for each treatment group. Each curve represents a treatment average based on multiple heifers with the solid and the liquid fractions for each treatment combined. Unique OTU's are estimated at a 10% difference level

Figure 5.2. Cluster analysis of dietary treatments created using Jaccard analysis to show dissimilarity among epithelial populations based on unique OTUs for each treatment. OTUs are estimated at a 10%.



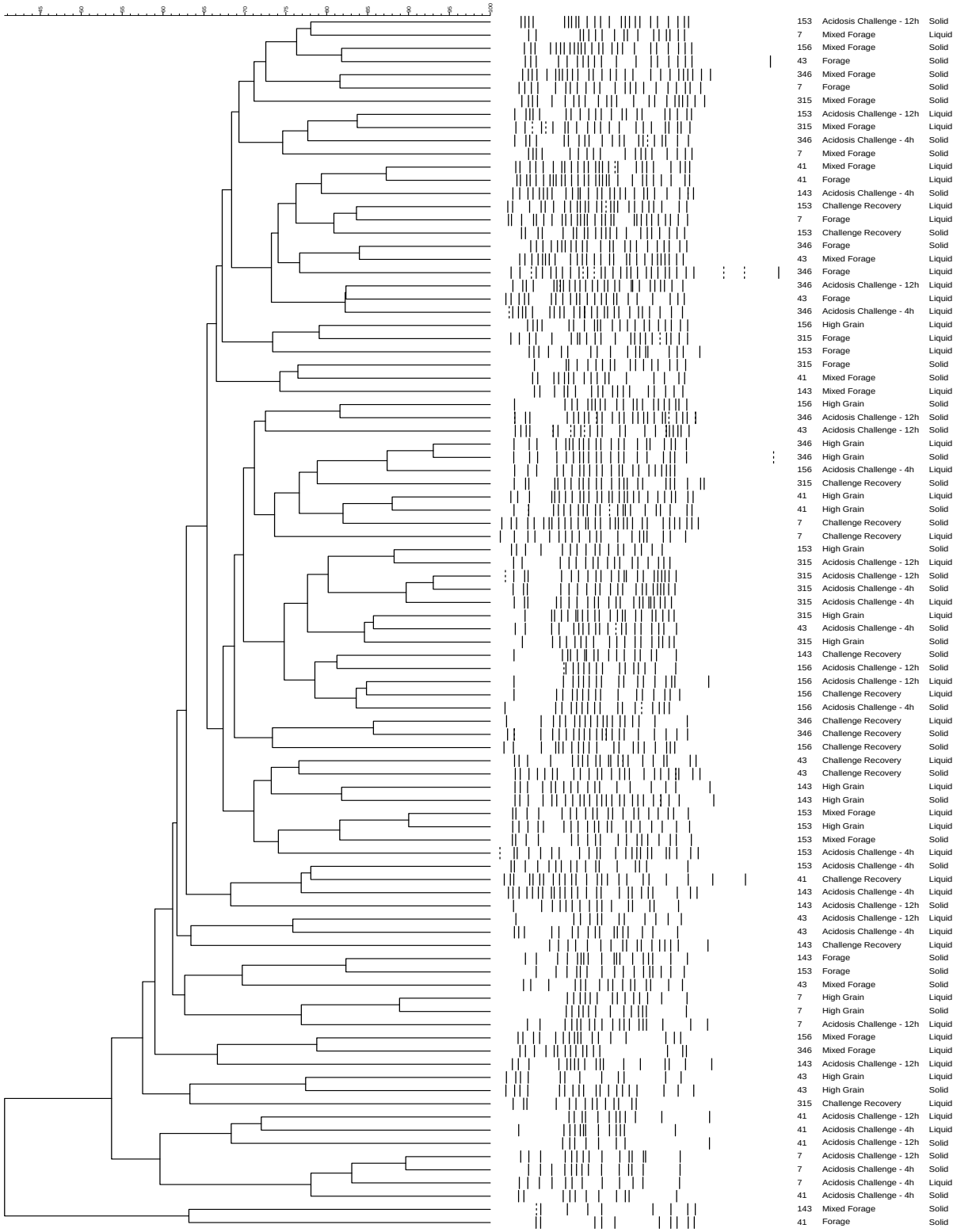
procedure of SAS (version 9.1.3; SAS Institute Inc., Cary, NC, USA). Using the same procedure, estimation of select species using real-time PCR relative quantification and rumen fermentation variables including VFA and pH were analyzed for effect of diet, heifer and interaction between heifer and diet. Percent taxonomic data were similarly analyzed after first being log-transformed (Duval *et al.* 2007) These data were first analyzed for heifer by diet interactions. Since no significant interactions were found, analysis was changed from a factorial to a block design to increase statistical power. Means were separated using Tukey's honest significant difference (HSD). All pH variables were additionally analyzed in a pairwise correlation to all unique OTUs. Significance was declared at $P \leq 0.05$; trends were indicated at $P \leq 0.10$.

5.3 Results

5.3.1 Bacterial community composition, abundance and occurrence

Microbial composition varied significantly among heifers fed the same diet (Figure 5.3). Using PCR-DGGE to compare the overall diversity fingerprint for each sample, cluster analysis showed no significant clustering of profiles based on individual heifer, diet or fraction of digesta (Figure 5.3). Real-time real-time PCR analysis of six different bacterial targets among each of the six treatments (forage, mixed forage, high grain, acidotic challenge 4h post-feeding, and 12h post-feeding and challenge recovery) and between the solid and liquid digesta are shown in Table 2. All of the bacterial targets, except for *S. bovis* were affected by diet. *Ruminococcus* spp. and *F. succinogenes* contributed to a larger percent of the total enumerated eubacteria for the mixed forage diet (18.09 and 3.64%, respectively) and contributed the least to the high grain diet (5.70 and 1.60%, respectively).

Figure 5.3. Dendrogram of PCR-DGGE analysis of rumen epithelial samples from cattle fed a progression of dietary treatments (forage, mixed forage, high grain, acidotic challenge and a challenge recovery). Clustered with Dice (Opt: 1.0%) (Tol: 1.0%-1.0%) and UPGMA.



The genus level target for *Prevotella* was highest in the acidotic challenge treatment at 4 h post-feeding and lowest in heifers fed the forage diet (Table 5.2). *Selenomonas ruminantium* and *M. elsdenii* contributed the least to the total enumerated eubacterial population with the forage diet (1.12 and 0.01%, respectively). The population of *M. elsdenii* was highest in heifers at 12 h post-feeding of the acidotic challenge and declined during the recovery period whereas *S. ruminantium* further increased during the post-challenge recovery. None of the real time real-time PCR targets were found to be significantly different between the solid and liquid digesta except for *F. succinogenes,* which was 1.23% higher in the solid-digesta (Table 5.2).

Percent relative abundance of all genera, as determined from analysis of pyrosequencing data were compared among heifers on each of the three major dietary regimes: forage, mixed forage and high grain using a heat map to display variation (Figure 5.4). This analysis found 72 distinct genera that varied in abundance between heifers, or between diets. Average number of sequences per heifer within diet and fraction ranged from 3,260 in the liquid fraction of the forage diet to 6,832 in the liquid fraction of the acidotic challenge treatment at 4 h post-feeding. The average number of sequences per heifer between the solid and the liquid digesta fractions were similar (data not shown). Percent abundance data obtained from pyrosequencing of all samples showed 59 individual genera that had either an effect of treatment, fraction or a treatment by fraction interaction (data not shown). A total of 35 genera and genus-level taxa including *12-18, Acidaminococcus, Adhufec405, Blautia, Fibrobacter, IS Eub hallii, IS Eub. rumin. Coprococcus A2 166, JW17, L7A-B08, L7B-A04, Lactobacillus, Marvinbryantia, P-1297-a5, Pannonibacter, Papillibacter, Prevotella, RC9, RF38, RFN63, RFN71, RFN8-YE57, Roseburia, Ruminobacter,*

Table 5.2. Effect of diet and digesta fraction on the percent of total enumerated eubacteria 16S rRNA genes of dominant rumen bacterial species using quantitative real-time PCR.

	Dietary Treatment[1]								**Digesta Fraction**[1]			
Bacteria	Forage	Mixed forage	High grain	Acidotic challenge 4h PF	Acidotic challenge 12h PF	Challenge recovery	SEM	*P*-value	Liquid	Solid	SEM	*P*-value
Ruminococcus spp.	8.01a	18.09b	5.70a	10.02a	6.75a	12.75a	1.820	<0.001	9.91	10.63	1.025	0.572
Fibrobacter succinogenes	2.86b	3.64b	0.16a	0.91a	0.57a	0.64a	0.489	<0.001	0.82a	2.05b	0.282	0.004
Prevotella spp.	2.86a	4.47ab	7.75bc	10.78c	7.22abc	8.26bc	1.120	0.002	7.67	6.29	0.644	0.118
Selenomonas ruminantium	1.12a	1.75ab	6.44cd	4.97bc	4.48bc	8.17d	0.725	<0.001	4.78	4.34	0.725	0.419
Megasphaera elsdenii	0.01a	0.18ab	0.43ab	0.50ab	0.69b	0.20ab	0.159	0.049	0.31	0.37	0.092	0.666
Streptococcus bovis	0.07	0.08	0.00	0.20	0.92	0.02	0.490	0.760	0.02	0.42	0.281	0.342

Significant Heifer×Treatment interactions for *Selenomonas ruminantium* and *Megasphaera elsdenii* (P<0.05). Significant Treatment x Fraction interactions for *Selenomonas ruminantium* (P<0.05).

PF: post-feeding

Figure 5.4. Heat map diagram of the relative percent abundance of all genera for each individual heifer (indicated by ID number) for each of the three dietary regimes: forage, mixed forage and high grain. Percent abundance increases with the darkness of the corresponding square from ≤1.0% to >15% of the total rumen population as averaged between the liquid and solid fractions.

Saccharofermentans, Selenomonas, Solobacterium, Sporobacterium, Sporotalea, Streptococcus, Succinivibrio, Thalassospira, Treponema, VadinHA24, and 12 taxa classified as uncultured were effected by diet. Of these, a significant effect of host was found for *12-18, Acidaminococcus, Adhufec405, Blautia, IS C. phytofermentans, L7A-B08, L7B-A04, P-1297-a5, Papillibacter, Prevotella, RC9, RFN63, Roseburia, Selenomonas, Solobacterium, Succinivibrio, Thalassospira, Treponema*, and *VadinHA42*. When comparing percent abundance between solid and liquid digesta, 9 of the 13 genera including *Atopostipes, Fibrobacter, IS Butyrivibrio, IS Eub. ruminantium coprococcus A2 166, Selenomonas, Sporotalea, Treponema, Wautersiella* and *Xylanibacter* were found to be highest in the solid-digesta.

5.3.2 Core microbiome

Determination of a core microbiome was done by comparing all samples for all heifers for both the solid and liquid digesta on all treatments. Any taxa found to be ubiquitous within all samples were then assigned as part of the rumen content core microbiome. Similar analysis was performed for each dietary treatment, comparing the solid and liquid-digesta from all samples from all heifers. When solid and liquid samples were combined for each heifer, analysis was done to determine which bacterial taxa were prevalent in whole rumen contents of all heifers on each of the three major dietary regimes (forage, mixed forage and high grain). From these data Venn diagrams were constructed (Figure 5.5 and 5.6). The overall core microbiome was found to consist of the phyla Bacteroidetes (32.8%), *Firmicutes* (43.2%) and *Proteobacteria* (14.3%). Both of the largest phyla had corresponding classes and orders that were found to be part of the core microbiome at slightly lower abundances (*Bacteroidia/Bacteroidales, Clostridia/Clostridiales*, Table 5.3). *Lachnospiraceae* and *Prevotellaceae* at the family level, as

well as *Prevotella* spp. at the genus level were also present in all samples. When individual treatments were analyzed for "core taxa", those heifers fed the forage treatment showed a distinctive core microbiome including 14 additional genera and two additional phyla *Spirochaetes* and *Fibrobacteres* (Table 5.3). The mixed forage core taxa showed numerically less total numbers of taxa compared to the forage and high grain diets. The phyla Proteobacteria and its corresponding class/order/family/genus *Gammaproteobacteria/ Aeromondales/ Succinivibrionaceae* / *12-18* were part of the core taxa of the high grain as was the phyla *Actinobacteria.* The total number of bacterial taxa present in the core microbiome for the acidotic challenge at 4 h and 12 h post-feeding was decreased compared to the previous diets (Table 5.3). Heifers recovering from an acidotic challenge differed minimally in their core taxa with only the addition of *Rikenellaceae* and *Gammaproteobacteria*, both of which had been part of the core taxa on previous dietary regimes.

5.3.4 Effects of acidotic challenge on rumen microbes

Individual animal pH response to the acidotic challenge has been previously reported in Chapter 4 (Table 4.12). In this study, it was shown that two of the eight heifers (7 and 41) used in the study showed the lowest mean daily pH (4.00 and 3.93, respectively) and the highest area under pH 5.2. Based on these parameters, heifers 7 and 41 were shown to have the most severe response to the acidotic challenge resulting in clinical acidosis. Whereas heifers 143 and 153 responded least severely to the challenge and were considered subclinically acidotic (Table 4.12). In the current study, the relative abundance of all genera found in the acidotic challenge samples, the percent difference in the clinical acidotic (7 and 41) and from subclinical acidotic (143 and 153) heifers was used to demonstrate the positive and negative impacts of

Table 5.3. Percent abundance of taxa to the "Rumen Core Microbiome" and the microbiome for forage, mixed forage, high grain, acidotic challenge (4 h post-feeding and 12 h post-feeding) and challenge recovery treatments.

		Dietary Treatments						
						Acidotic challenge		
Taxa Level	Classification	Core	Forage	Mixed forage	High grain	4 h PF	12 h PF	Challenge recovery
Phyla	*Actinobacteria*				1.6		1.4	
Phyla	*Bacteroidetes*	32.8	25.7	26.2	40.3	40.0	34.5	31.5
Class	*Bacteroidia*	31.1	24.1	24.9	37.7	37.8	33.6	29.8
Order	*Bacteroidales*	31.1	24.1	24.9	37.7	37.8	33.6	29.8
Family	*S24-7*		3.8	2.2				
Genus	*RC9*		3.3	2.7				
Family	*Prevotellaceae*	24.7	12.0	16.3	32.7	33.2	29.9	25.5
Genus	*Prevotella*	22.2	8.9	12.8	31.6	30.3	28.3	24.1
Genus	*12-129*		1.6					
Genus	*BF34*		1.2					
Family	*Rikenellaceae*		3.7	3.0				1.4
Family	*BS11*		2.3					
Phyla	*Cyanobacteria*				1.8			
Class	*4C0d-2*				1.7			
Phyla	*Fibrobacteres*		7.1					
Class	*Fibrobacteria*		7.1					
Order	*Fibrobacterales*		7.1					
Family	*Fibrobacteraceae*		7.1					
Genus	*Fibrobacter*		7.1					
Phyla	*Firmicutes*	43.2	55.2	55.8	37.0	33.6	37.2	43.7
Class	*Erysipelotrichi*		1.8					
Order	*Erysipelotrichales*		1.8					
Family	*Erysipelotrichaceae*		1.8					
Class	*Clostridia*	40.5	53.3	53.9	34.9	31.1	32.0	41.7

		Dietary Treatments						
						Acidotic challenge		
Taxa Level	Classification	Core	Forage	Mixed forage	High grain	4 h PF	12 h PF	Challenge recovery
Order	*Clostridiales*	40.3	53.1	53.4	34.5	31.0	31.9	41.5
Family	*Ruminococcaceae*		17.3					
Genus	*Papillibacter*		1.3					
Genus	*RFN71*		1.6					
Genus	*Ruminococcus_1*		1.7					
Genus	*SP3-e02_2*		0.9					
Genus	*Saccharofermentans*		2.4					
Genus	*vadinHA42*		4.0					
Family	*Lachnospiraceae*	19.3	32.1	22.7	16.9	12.2	15.0	18.3
Genus	*Acetitomaculum*		0.9					
Genus	*Butyrivibrio_*		2.3					
Genus	*RFN8-YE57*		10.9					
Family	*Clostridiaceae*		0.9	1.2				
Phyla	*Proteobacteria*	14.3	4.7	8.9	17.9	16.5	20.1	15.2
Class	*Gammaproteobacteria*				14.7	14.8	17.9	12.6
Order	*Aeromonadales*				14.3	14.5	17.7	
Family	*Succinivibrionaceae*				14.3	14.5	17.7	
Genus	*12-18*				7.2			
Class	*Alphaproteobacteria*		2.3			1.3		
Phyla	*Spirochaetes*		2.8					
Class	*Spirochaetes*		2.8					
Order	*Spirochaetales*		2.8					
Family	*Spirochaetaceae*		2.7					
Genus	*Treponema*		2.7					

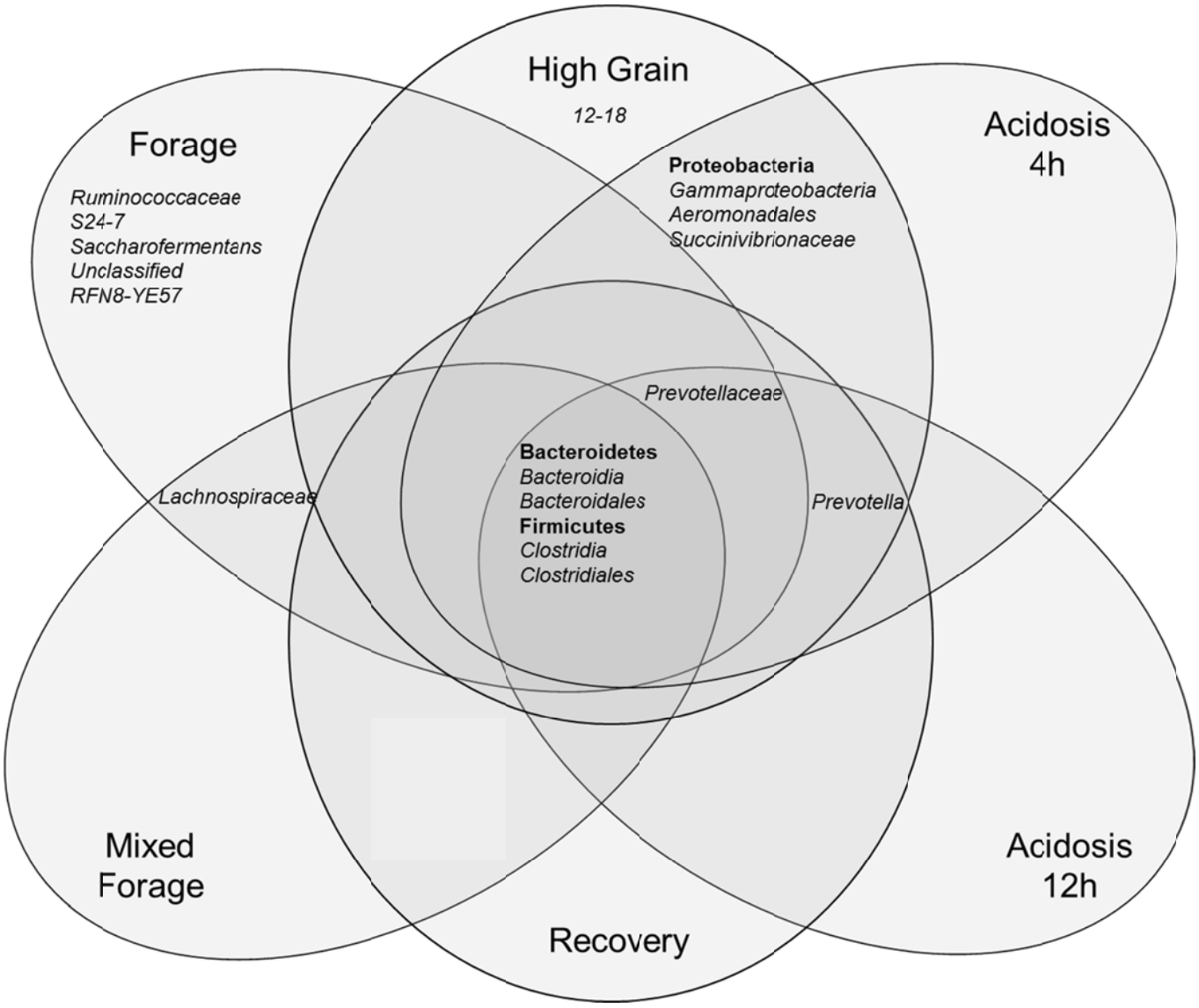


Figure 5.5. Venn diagram of the rumen dietary core microbiomes as determined by only those taxa which were ubiquitous for the solid and liquid-digesta in all heifers. Each circle represents a treatment; bacterial taxa within overlapping areas were common to the corresponding treatments.

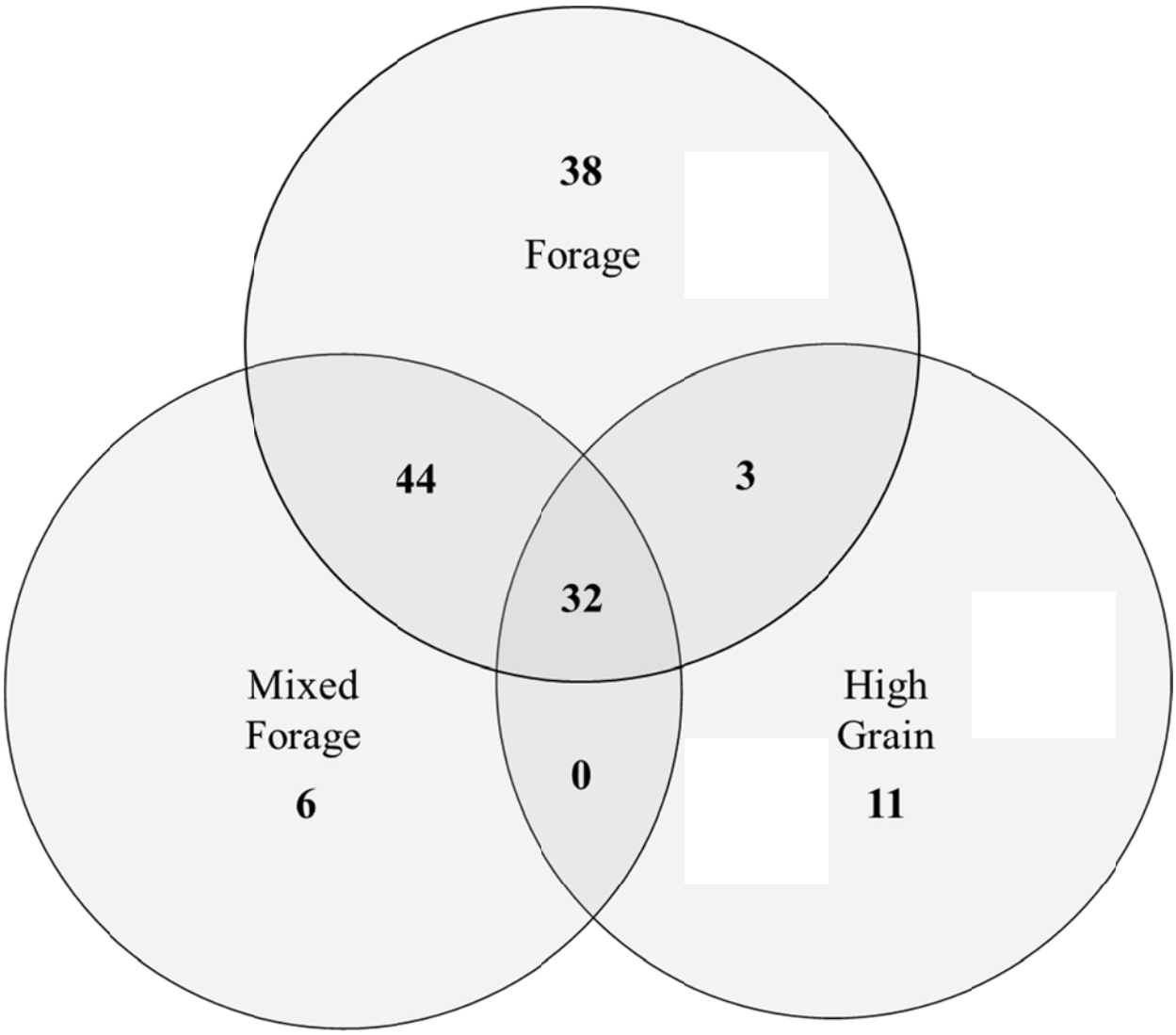


Figure 5.6. Venn diagram of the rumen core microbiome as determined by the presence of taxa in whole rumen contents, in all heifers. Each circle represents each of the major dietary treatments, numbers within circles or overlapping areas indicate the number of OTU's were common to the corresponding dietary treatments

an acidotic challenge on the bacteria of the combined liquid and solid- rumen contents (Figure 5.7). *Prevotella* exhibited the greatest change between clinical and subclinical acidotic groups with more than 26% increase at 4 h post-feeding and an 11% increase 12 h post-feeding in the clinically acidotic heifers (Figure 5.7). *Acetitomaculum, L7A B08, Pseudobutyrivibrio, Selenomonas,* and *vadinHA42* all increased at 4 h post-feeding, in the clinically acidotic heifers and the percent difference increased even further at 12 h post-feeding, with the most dramatic increase being in *Acetitomaculum* (11% increase; Figure 5.7). *Lactobacillus* and *Streptococcus* were the only bacteria, which were not present at 4 h post-feeding but were greatly increased in the clinically acidotic heifers at 12 h post-feeding (3.7% and 3.4%, respectively). The most dramatic increase in the percent abundance at the genera level was found in those heifers that were subclinically acidotic including unidentified rumen bacterium "*12-18",* as well as *Succinivibrio,* and *Treponema* (10.8%, 12.3% and 9.6% higher at 4h post-feeding, respectively). Of these, only "*12-18"* increased in percent difference from 4 h post-feeding to 12 h post-feeding. Though a number of greater genera were found to be higher in the subclinically acidotic heifers, most of this increase was less than 3%. *RFN8-YE57* exhibited the most dramatic change between 4 h post-feeding and 12 h post-feeding being 2.4% higher in the clinically acidotic heifers and increasing to 4.5% higher in the subclinically acidotic heifers 8 h later (Figure 5.7).

A correlation analysis of the key pH parameters (pH min and pH area under 5.2) to all classified genera was performed and 6 genera were correlated to one or more pH parameters in the solid and liquid-digesta samples (Table 5.4). *Prevotella* increased (<0.001) in the solid and liquid rumen contents as minimum pH decreases and pH area less than 5.2 increased.

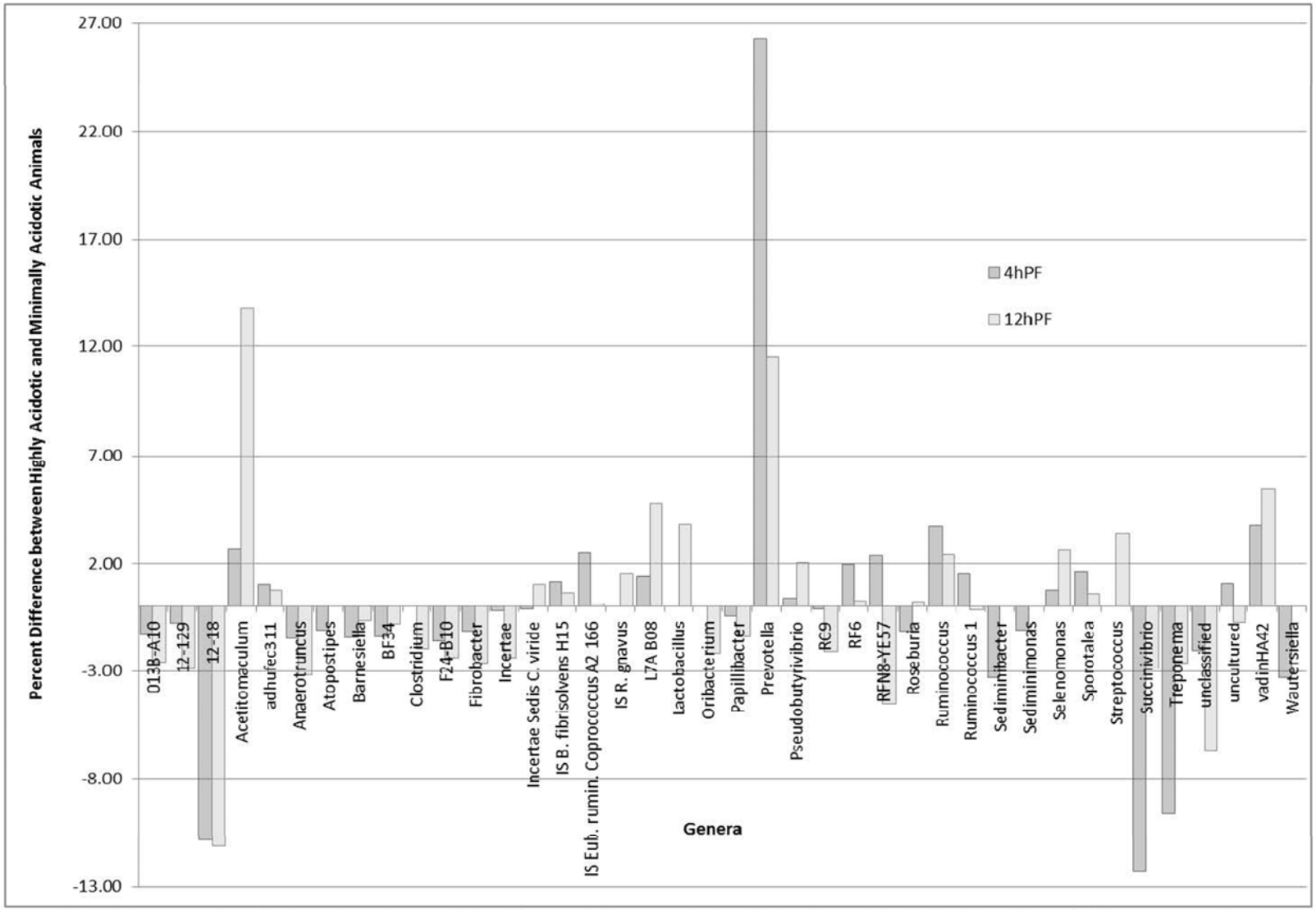


Figure 5.7. Difference in relative abundance (%) of bacterial genera's in clinically acidotic heifers (7 and 41) compared with heifers that were subclinically acidotic (143 and 153) at both 4 h and 12 h post-feeding. acidotic ranking was determined by area under the curve for pH<5.2 adjusted for DMI (data not shown).

The liquid and solid digesta associated *RFN8-YE57* responded ($P \leq 0.05$) to pH, decreasing in total number as pH decreased or pH area less than 5.2 increased. Other *genera* that also decreased as daily mean pH decreased included *IS Eub. hallii,* and *vadinHA42*. Conversely, *Acidaminococcus,* and *IS Eub. rumin Coprococcus A2166I* increased in percent abundance as pH minimum decreased. All *genera* which increased in response to lowest mean pH, also increased as pH area under 5.2 increased.

5.4 Discussion

The majority of the current knowledge of the rumen microbiome has been derived using traditional culturing and enumeration methods (Hungate 1969; Dehority *et al.* 1989). However, as many microbes cannot be cultured, and those which can represent such a small fraction of the total microbiome (Amann *et al.* 1995; Krause and Russell 1996; Shin *et al.* 2004; Deng *et al.* 2007), newer methods are needed to help further elucidate the dynamics of the rumen ecosystem. Molecular techniques based on 16S rRNA (Kong *et al.* 2010b) can not only be used to predict evolutionary relationships without the need to cultivate organisms, they can also be used to enumerate microbes (Mackie *et al.* 2003; Kobayashi 2006; Deng *et al.* 2007). Next generation sequencing of total DNA from environmental samples has revealed the complexity of many gut microbial communities, highlighted individual animal variability, and characterized imbalances in the gut microbiome and its implications for host health in humans, chickens and cattle (Ley *et al.* 2006b; Turnbaugh *et al.* 2006; Malmuthuge *et al.* 2012). Previous estimates have shown that 80,000 sequencing reads should be sufficient to cover all the OTUs in the rumen (Kim *et al.* 2011; Jami and Mizrahi 2012). This estimate is supported by the rarefaction curves developed for each dietary treatment in this study.

Table 5.4. Correlation of key pH parameters to rumen genera in both the solid and liquid-digesta of heifers subject to an acidotic challenge.

		pH Parameters			
		pH min		pH area under 5.2 (phxmin)	
Taxon		S*	L*	S	L
Acidaminococcus	Correlation	-0.30	-0.36	0.11	0.19
	P-Value	0.11	0.05	0.56	0.32
IS Eub. hallii	Correlation	0.45	0.31	-0.29	-0.33
	P-Value	0.01	0.09	0.13	0.07
IS Eub. rumin Coprococcus A2 166	Correlation	-0.32	-0.38	0.35	0.48
	P-Value	0.08	0.04	0.06	0.01
Prevotella	Correlation	-0.61	-0.68	0.56	0.61
	P-Value	<0.001	<0.001	<0.001	<0.001
RFN8-YE57	Correlation	0.58	0.60	-0.31	-0.36
	P-Value	<0.001	<0.001	0.10	0.05
vadinHA42	Correlation	0.27	0.38	-0.10	-0.23
	P-Value	0.15	0.04	0.61	0.23

* Solid and liquid fractions of digesta are indicated by S and L, respectively.

Though 80,000 sequencing reads is potentially excessive for the seemingly less diverse diets containing predominantly grain, it is clear that the diversity of forage-based diets (forage and mixed forage) require a significantly greater sequencing depth in order to obtain full coverage of the rumen microbiome. Jami and Mizrahi (2012) were able to sequence up to a maximum of 16,000 sequences per sample in mature dairy cow whereas Li *et al.* (2012a) sequenced 30,000 reads per sample in the pre-ruminant calf. In the current study, 21,000 to 36,000 sequences were determined for each of the dietary treatments. Since a key aspect in understanding any ecosystem is to first identify its permanent and temporary residents, the aim of this study was to expand on the body of rumen microbiological research by characterizing the rumen solid and liquid-digesta associated bacterial communities in heifers fed forage, mixed forage-grain and high grain diets as well as during and after recovery from an acidotic challenge. Additionally, this study aimed to further elucidate a potential 'core microbiome' in the rumen and determine if individual animal susceptibility to an acidotic challenge could be correlated to changes in the rumen microbial ecosystem.

Although individual host variability is a well-known (Costello *et al.* 2009; Tap *et al.* 2009) and a documented concept in human intestinal microbiology, evidence indicates that a similar relationship exists in ruminants (Mohammed *et al.* 2012;Weimer *et al.* 2010). Modern molecular analysis have assessed individual animal variation using fingerprinting techniques such as PCR-DGGE (Martinez *et al.* 2010) and automated ribosomal intergenic spacer analysis (ARISA; Mohammed *et al.* 2012; Weimer *et al.* 2010). However, these techniques are limited because they cannot provide information at the genera and species taxa levels. Despite this limitation, these studies have been able to clearly show that each host has unique components within its microbial community. In this study, the detailed analysis of metagenomic sequencing

data were able to reveal host variation in the total number of sequences, the total number of unique OTU's identified for each heifer, the richness and diversity indices and identify a large number of distinct genera (Table 5.1; Figure 5.4). These data confirm that there is a large amount of individual host variation in rumen microbial ecosystems. This conclusion was further supported by the large variation found in the PCR-DGGE analysis performed in this study (Figure 5.3).

In the past few years, human gastrointestinal microbiology has largely focused on elucidating the 'core microbiome', those species that are found in every individual (Ley *et al.* 2006a; Turnbaugh *et al.* 2007; Tap *et al.* 2009) and this concept has also recently begun to be applied to rumen microbial ecology (Jami and Mizrahi 2012; Li *et al.* 2012b). However, previous studies in ruminants were done using only one dietary regime as the basis for their analysis. One of the largest barriers to determining the core microbial population in humans is the diversity of dietary composition based on culture, location and individual food preferences (Turnbaugh *et al.* 2007). While each dietary regime can have its own distinct microbial profile, the true 'core microbiome' is present regardless of dietary composition (Tschop *et al.* 2009; Muegge *et al.* 2011). In cattle, dietary composition is diverse and based on a number of factors. However, it is easier to control and accurately analyze as compared to similar research with humans and therefore the determination of a 'core microbiome' for cattle is even more feasible. Jami and Mizrahi (2012) were able to identify 32 genera across 16 cattle whereas Li *et al.* (2012b) identified 26 genera that were common in 4 cattle used in their study. Unlike previous research, the current study was only able to identify a single genus, *Prevotella* (22.2%), which was ubiquitous to all samples and therefore defined as part of the core rumen microbiome. However, at higher taxa levels, the rumen core microbiome was elucidated (Table 5.3). The major

discrepancy between the findings of the current and previous studies are most likely due to the diversity of dietary regimes analyzed in our study. Both previously published studies used a diet of between 50 and 70% concentrate with the remainder of the diet being forage. The presence and metabolic importance of *Prevotella* spp. in the rumen has long been recognized (Bryant and Burkey 1953; Hungate 1966; Bryant 1970; Stevenson and Weimer 2007; Jami and Mizrahi 2012). Classical microbiology indicated that *Prevotella* were proteolytic and while many species of this genus have the capacity to degrade protein, this genus is present in the rumen across a variety of diets suggesting that it has a broader nutritional diversity than originally supposed (Stevenson and Weimer, 2007).

The wide variety of diets employed in the current study more accurately defined the core microbiome compared to previous studies as a range of forage through high concentrate diets was used in the profiling of key ruminal bacteria (Jami and Mizrahi 2012). By determining the rumen core microbiome, this research has provided a basis for which to compare the key changes in the rumen microbial ecosystem under different dietary conditions and during perturbations such as acidosis (Tschop *et al.* 2009; Li *et al.* 2012b; Mohammed *et al.* 2012). Acidosis is one of the most potent onslaughts to the stability of the rumen microbial population, by first outlining the core microbiome in the rumen and then documenting the impact of clinical and subclinical acidosis we are able to clearly show the impact of acidosis on the rumen microbial community and determine that clincial or subclinical acidosis does not cause a perpetuated change in the rumen microbiome. Furthermore, we were then able to determine if the rumen microbial ecosystem reverts back to those populations that were present in the pre-challenge state.

Rumen bacteria have been classified into three major compartments according to their spatial location within this environment, free-living bacteria associated with the liquid digesta,

adherent bacteria associated with the feed particles and the epimural community which is adherent to the rumen epithelial tissue (Wallace *et al.* 1979; McAllister *et al.* 1994; Cheng and McAllister 1997). Despite the clear delineation between these rumen content associated populations, most studies analyze pooled samples of liquid and solid-fractions and relatively few molecular based studies have looked at the differences between these populations under various dietary regimes (Kong *et al.* 2010b; Pitta *et al.* 2010; Hess *et al.* 2011b; Petri *et al.* 2012). Data using real-time PCR methodology showed that of the quantified bacterial primers, only *F. succinogenes* was significantly higher in the solid versus the liquid digesta. Furthermore, only 4 genera were significantly different between digesta fractions without any dietary interactions. *Wautersiella, IS Eub rumin Coprococcus* and *IS Butyrivibrio* were significantly higher in the solid fraction and *Atopostipes* was highest in the liquid digesta. The predominance of the first three genera in solid digesta likely indicates that these bacteria are members of the feed adherent biofilm. Recent pyrosequencing research of the rumen microbiome found a number of bacterial groups that were associated with solid digesta including members of *Spirochaetaceae* and *Fibrobacteraceae*, as well as the genera *B. fibrisolvens* (de Menezes *et al.* 2011; Fouts *et al.* 2012). The presence of *Atopostipes* in only the liquid digesta may indicate that this genus is not a direct component of the digesta biofilm and may solely be a liquid associated bacterium. While supporting data for these genera are unavailable it has been previously identified and characterized in aquatic environments and therefore may have similar metabolic roles in the rumen as it does in other environments.

It has also long been understood that diet influences the diversity and community composition of rumen contents (Tajima 2000; Kocherginskaya 2001; Tajima *et al.* 2001; Firkins *et al.* 2008) and studies using a variety of molecular techniques have been able to elucidate the

changes associated with dietary changes and subclinical ruminal acidosis (Kocherginskaya *et al.* 2001; Fernando *et al.* 2010; Kong *et al.* 2010a; Khafipour *et al.* 2011). This experiment further expanded on this body of knowledge by providing additional detail in analyzing the liquid and solid associated rumen bacterial fractions of heifers fed a variety of dietary treatments. The specificity of 454 pyrosequencing data were able to classify over 44 distinct genera that varied significantly based on dietary composition or rumen fraction. Several of these bacteria are among the most commonly researched ruminal bacteria including *Fibrobacter, Prevotella, Rumminococcus, Selenomonas, Streptococcus* and *Succinivibrio.* Some of the genera identified in this study have also been found in other culture-dependent and independent studies including *Treponema* (Bryant 1970; Kocherginskaya *et al.* 2001; Koike *et al.* 2003; Pitta *et al.* 2010) and *Ruminobacter* (Dehority 1969; Tajima *et al.* 2001). In this study, a number of genera previously unreported in the rumen were identified as sensitive to diet, fraction or the interaction of these two factors including *Atopostipes, Pannonibacter, Persicitalea* and *Thalassospira*. While all of these genera have been previously identified as proteobacteria from aquatic environmental samples, only *Atopostipes* has been associated with the gastrointestinal tract (Cotta *et al.* 2004). While the inability to describe the metabolic and functional capacities of a large number of the bacterial taxa identified in pyrosequenced rumen samples limits our understanding of this ecosystem, detection and identification are the initial steps required in order to progress our knowledge in this field.

Hungate was the first to study alterations in the microbiome of the rumen to explain the "microbial actions" causing digestive disturbances in sheep and cattle. He reported that an excess of grain introduced into the rumen caused the cellulolytic bacteria to greatly decrease in numbers while the relative numbers of Gram-positive bacteria increased (Hungate *et al.* 1952). This study

also presented evidence that *S. bovis*, a gram-positive bacterium, plays an important role in ruminal acidosis. These initial observations on major microbial alterations during ruminal acidosis remain valid. However, there is still little known about the microbial changes associated with subclinical acidosis (Goad *et al.* 1998; Nagaraja and Titgemeyer 2007; Khafipour *et al.* 2009). Comparison of the core taxa present during acidotic challenge to the core rumen microbiome shows those population changes that maybe indicative of this condition. The biggest differences between these two rumen profiles were observed for Proteobacteria and *Firmicutes* populations. Increases were seen in the *Proteobacteria* from 14.3% in the core microbiome to as much as 20.1% in the acidotic challenge at 12 h post-feeding. Whereas the phylum *Firmicutes*, a gram-positive group, was decreased by up to 10% at 4 h post-feeding compared to the core microbiome. This is contradictory to Hungate's proposal that the relative numbers of gram-positive bacteria increased under acidotic conditions (Hungate *et al.* 1952). While the details of these changes were unidentifiable at the genus level in this study, these data provide a basis for further research into the core taxa associated with an acidotic challenge. Furthermore, Hungate *et al.* (1952) stated that numbers of cellulolytic bacteria were greatly decreased as acids accumulated in the rumen, whereas our study shows that the core microbiome has minimal cellulolytic bacteria present and therefore decreased numbers of cellulolytic bacteria such as *Ruminococcus* occurred with the high concentrate diet, suggesting that their decline occurs without the host experiencing acidosis..

By comparing diet sensitive bacterial genera from heifers which showed a severe response (clinical acidosis) to an acidotic challenge with those which responded minimally (subclinical acidosis), this study was able to identify a number of critical bacteria associated with acidotic ruminal conditions. Similar to the original findings by Hungate *et al.* (1952), *Streptococcus* spp.

were found to be prolific under acidotic conditions, as were *Lactobacillus* spp. and *Selenomonas* spp. Population increases in a number of other genera as a result of acidotic conditions included *Acetitomaculum, L7A-B08, Pseudobutyrivibrio* and *vadinHA42*. Of these genera, only *vadinHA42* has not been previously identified in the rumen. While *Pseudobutyrivibrio* has been identified as belonging to *Clostridium Cluster* XIVa, the exact metabolic characteristics of these genera remain unknown and therefore warrant further exploration. Also in this study it was been noted that *Prevotella* and *Succinivibrio* are responsive to perturbations in the rumen environment, increasing in percent abundance under conditions such as acidosis (Li *et al.* 2012). However, due to the ubiquitous presence of *Prevotella* spp. in the rumen as part of the core microbiome, these changes may be simply be due to changes in nutrient availability. Analysis of the post challenge recovery period was done to determine the recovery potential of the core microbiome. However, during the challenge recovery period heifers showed no unique species indicative of a modified post-challenge core taxa (Figure 5.5). The only change in the core taxa from the acidotic challenge was an increase in Rikenellaceae spp., which was previously only found in heifers consuming forage. The family Rikenellaceae has been previously found in the digestive tracts of cattle (Kong *et al.* 2010b) fed triticale and is commonly found in digestive tract environments. The metabolic function and role of this family in the rumen microbiome requires further research. The ability of the rumen microbiome to recovery after perturbation, such as the addition of probiotics or the replacement of rumen contents, has been previously documented (Mohammed *et al.* 2012; Weimer *et al.* 2010). These studies showed that despite ruminal content replacement (Weimer *et al.* 2010) or acidotic challenge (Mohammed *et al.* 2012) the core microbiome was robust and resistant to change. Other studies have shown transient establishment of cellulolytic bacterial strains in the rumen, but it required repeated dosing, an

undeveloped ruminal microbiome, or other forms of selective pressure (Krause *et al.* 1999; Chiquette *et al.* 2007; Paul *et al.* 2007). Long-term physiological effects have been noted in animals challenged with acidosis such as greatly reduced feed intake (Brown *et al.* 2000) reduced ruminal VFA absorption and decreased liquid passage rates (Krehbiel *et al.* 1995). However, those animals with reduced feed intake did eventually recover and those animals with permanent changes in the rumen microbial profiles were lambs that may have had an underdeveloped ruminal bacterial ecosystem. Our results show that rumen microbiome returned to pre-challenge states in all heifers within a week of an acidotic challenge, regardless of whether the host was clinically or subclinically acidotic. Therefore, it is likely that, similar to the research in piglets, there is a limited window of time during early development that the core microbiome can be altered (Kelly *et al.* 2006). Perturbations to the rumen core microbiome outside of this early development window would then be mostly transient with a tendency to return to the pre-perturbation state (Weimer *et al.* 2010).

Ruminal pH has important implications for the microbial community composition, often resulting in an undesired population shift that results in inefficient digestion of feedstuffs (Russell *et al.* 2009; Li *et al.* 2012b). Variability in ruminal pH among cattle has long been acknowledged, but not understood (Bevans *et al.* 2005; Nagaraja and Titgemeyer 2007; Penner *et al.* 2009). A correlation analysis was performed on key pH parameters (pH min and pH area under 5.2) to all identified genera and while it can be determined which bacteria increased/decreased as rumen pH decreased and the total area under pH 5.2 increased, the data can only allow us to determine the pH sensitivity of the corresponding genera and not the metabolic functions which are responsible. .

5.5 Conclusions

The development and advancement of molecular techniques and their use in complex ecosystems such as the rumen has reinitiated investigations into the basic rumen microbial ecology questions from 50 years ago. Mainly, what bacteria species are present in the rumen and what is their role in the rumen ecosystem. Though we are still unable to fully answer these questions, the development of next generation sequencing and renewed sequencing efforts in the Hungate 1000 project ensure that progress is being continually made. Molecular analysis of the rumen microbiome has made it clear that there are host-microbe interactions, that each host has a partially unique rumen microbial population and that there is a rumen core microbiome shared by all hosts regardless of diet. This experiment clearly defined that rumen core microbiome and showed that it is stable during clinical acidosis. Furthermore, it was shown that the rumen core microbiome can mostly recover from such a perturbation within a short time. The resilience of the rumen core microbiome is likely due in part to the host-microbe interactions, as seen in other mammals such as humans and pigs. Further research is needed to determine if there is a window for microbial programming or alteration of rumen microbial succession in early rumen development to alter the rumen microbiome to increase animal productivity, feed efficiency or potentially reduce nitrogen and methane excretion. Such an experiment would require looking at microbial development of the rumen from birth through to maturity to determine when stability of the ecosystem is reached and at which points through that period the microbiome can be changed by the addition of bacteria to the system.

CHAPTER 6
6.0 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

The objective of the first experiment was to study the impact of the removal of forage from a high grain diet on rumen bacterial populations using molecular techniques. No clustering was detected in the analysis of PCR-DGGE fingerprint profiles, indicating that despite changes in diet ingredients, dietary NDF remained similar and therefore fermentability of both diets was still quite similar. Despite the change to DGGS replacing all silage and some barley grain, both diets were similarly low in effective fibre and considered highly digestible. Large variation that was seen in the PCR-DGGE profiles was also seen in the daily pH profiles of individual heifers. Animal variability is a significant factor in rumen microbial and pH analysis; it is well researched and is believed to be reflective of the differing abilities of individual animals in the production and absorption of VFA's (Brown *et al.* 2000, Bevans *et al.* 2005; Penner *et al.* 2009).While previous studies have shown diet clustering in comparison of forage vs. concentrate diets (Kocherginskaya *et al.* 2001), others have found high individual animal variation and animal specific clustering (Li *et al.* 2009). High variability in individual animal pH and VFA concentration, especially in highly fermentable diets such as those used in this experiment may have masked treatment effects on bacterial populations as determined using PCR-DGGE analysis.

The readily digestible nature of the fibre found in DDGS may have provided fibrolytic bacteria with fermentable substrate, even with barley silage removed from the diet. However, despite the highly digestible fibre in DDGS, the prevalence of *F. succinogenes* as measured using real-time PCR analysis showed a 57-fold decrease in cattle fed the no-forage diet.

However, previous cultivation studies have shown that *F. succinogenes* adheres to the most fibrous components of the diet (Halliwell and Bryant, 1963; Koike and Kobayashi 2009) which may have been decreased in DGGS components due to the processing required in making this feedstuff. However, even though *F. succinogenes* and other cellulolytic species such as *Ruminococcus* spp. decreased in the no-forage diet, these species did not completely disappear. This supports the theory that the readily digestible fibre of DDGS still acts as a substrate for cellulolytic bacteria to grow despite the lack of physically fibrous cell components such as silage on which this bacteria prefer to form biofilms.

The use of the molecular technique PCR-DGGE to study the rumen bacterial community under dietary changes showed that bacterial diversity was not significantly increased or decreased when DDGS substituted forage and some grain within the diet, regardless of the impact on rumen pH. Quantitative real-time PCR analysis clearly showed that key cellulolytic species decreased when forage was removed from the diet, but were still detectable despite the lack of preferred substrate. Therefore it is possible to deduce that the detectable bacterial community in the rumen is highly diverse and even reducing diet complexity and decreasing pH by removing forage did not decrease this diversity. However, we were unable to determine whether the associative effects of substitution of grain with forage on the rumen bacterial community reflect a change in density of bacterial groups without impacting diversity. The results of this experiment indicate that while changes in the rumen ecosystem do occur with a change in substrate, the stability and diversity of the ecosystem is maintained.

The objectives of the second study were broken into two parts. The first objectives were to characterize the composition of the epithelial-adherent bacterial community during dietary

progression from an all-forage to a barley grain-based diet with further monitoring during and after an acidotic challenge with the objective of defining indicator epithelial bacterial populations of acidosis. The second set of objectives was to identify and enumerate the liquid and solid-phase associated rumen bacterial communities during the same dietary progression and acidotic challenge. Bacterial populations were profiled and compared among individual hosts that were categorized based on the severity of their response to the acidotic challenge.

Classical microbial analysis of the rumen epimural community showed that this ecosystem contains a significant number of Gram-positive bacteria that are impacted by dietary changes (Cheng *et al.* 1980; Dinsdale *et al.* 1980; Wallace *et al.* 1979). In order to validate these data using current molecular technique analysis of the rumen under dietary transition from forage to high grain and then during and after an acidotic challenge was done using PCR-DGGE, real-time PCR and pyrosequencing. Polymerase chain reaction-DGGE profiles did not cluster and diversity analysis (Shannon and Simpsons indices) and species richness calculations (Chao1 and ACE) indicated no change in the populations regardless of diet or acidotic challenge. This indicated that epimural and digesta-liquid associated bacterial communities of individual heifers was highly stable. Of the 6 real-time PCR primer sets *F. succinogenes* was the only one which was found to vary based on diet composition, which matches the results of the previous experiment. When comparing diets using pyrosequencing data, differences were only seen at the genus level and on average represented less than 5% of the total sequenced bacteria. The low total numbers of bacteria which were impacted by the diet adds further support to the idea that the rumen epimural community is stable, with only slight shifts in the bacterial community as a result of diet change.

Pyrosequencing analysis of the rumen epithelial attached bacterial populations found 166 distinct species belonging to 61 genera, the majority of which were previously unidentified in the rumen epimural community. This included the genera *Azonexus, Filifactor, Marvinbryantia, Sharpea, Solobacterium, Thermodesulfobium* and *Thermohalobacter*. Many of these belong to the diverse class Clostridia, which is known to be associated with the rumen epithelial despite a lack of understanding in the metabolic role this group plays in the epimural community. The remaining genera including *Azonexus* and *Thermohalobacter,* are part of the Proteobacteria phyla and belong to families, which have been previously identified within rumen systems (Yang *et al.* 2010; Chen *et al.* 2011). Perhaps the most interesting find in the epimural community was the presence of *Solobacterium*. This genus from the *Erysiopelotrichales* order have only been described as part of the gut microbiome in a number of mammals including humans since the application of molecular techniques to gut ecosystems (Kageyama and Benno, 2000; Morita *et al.* 2008; Somer and Summanen, 2002). Analysis of all samples showed that *Solobacterium* were part of the core microbiome of the rumen epimural community in the present study making them a new major population in rumen microbial ecology.

Recently, the concept of a core microbiome was applied in lactating cattle (Jami and Mizrahi, 2012) by pyrosequencing bacteria extracted from rumen solid digesta from a single diet. This experiment attempted to show a core microbiome that was more representative of the rumen ecosystem of cattle on a wider variety of diets and from the epithelial bacteria, which is potentially more essential to the rumen than that of digesta-associated biofilms due to the fact that the enzymatic activities of this ecosystem are often integrated with those of the tissue itself.

When analyzing the core microbiota of the solid and liquid fractions of the rumen our research was only able to identify one genera-level taxa, *Prevotella* (22.2%), as part of the rumen

core microbiome whereas Jami and Mizrahi (2012) were able to identify 21 genera (32 taxa) across 16 cattle and Li *et al.* (2012b) identified 26 genera that were common to all 4 cattle used in their study. The major discrepancy between the findings of the current study and those previous is likely due to the diversity of diets analyzed in the current study. When looking at only those samples on the Mixed Forage diet which most closely matched previous studies, 16 genera were found to be in all 8 heifers and of these only 7 were identified as known cultured organisms. Despite a lack of diversity found in the core microbiome over a variety of diets, this study has identified which taxa are likely quintessential rumen bacteria.

The results of pyrosequencing analysis of the rumen digesta contents in this study, when compared to the classical microbiological analysis of the rumen showed a few key differences. Phylogenetic reclassification of a number of bacteria and the identification of a greater number of bacterial species within the past 50 years resulted in bacterial groups with different names and the use of DNA sequence comparison may have resulted in greater detail in describing morphologically similar bacteria. Classical work by Cheng and Wallace (1979) identified populations of *Micrococcus*, *Corynebacterium* and *Propionibacterium,* whereas in the present study only *Atopobium* and *Olsenella* were identified. Members of the genus *Atopobium* and *Olsenella* are closely related and have only recently been identified through the molecular characterization of the microbial epimural community of the rumen (Chen *et al.* 2011; Cho *et al.* 2006; Li *et al.* 2012).

Of all dietary regimes, those which have the potential to result in acidotic conditions have been shown to elicit the greatest variation in pH response and perturbation of the rumen ecosystem. Some individuals, called non-responders, are able to withstand the negative impacts of a ruminal acidotic challenge and others undergo severe repercussions from a similar challenge

(Penner *et al.* 2009). When comparing epithelial samples high levels of *Lactobacillus* were found in the rumen of 6 out of the 8 heifers during the acidotic challenge which agrees with previous work on adherent populations in the gastrointestinal tracts of many mammals including ruminants and humans (Edwards *et al.* 2004; Heilig *et al.* 2002; Pedersen and Tannock, 1989). However, lactobacilli in this study were only found during the acidotic challenge. Furthermore, *Lactobacillus* was measured at levels 16% higher in those individuals (7 and 41) that responded most severely (pH min ≤ 4.0) indicating a correlation between clinically acidotic conditions and the presence and abundance of *Lactobacillus*. Clinically acidotic heifers (7 and 41) also had the only detectable *Streptococcus* populations during the acidotic challenge. Previously, *Streptococcus* spp. have been understood to be a key bacterium in the "acidosis spiral" theory (Heilig *et al.* 2002; Pedersen and Tannock, 1989). This theory proposes that *Streptococcus* persists at low levels in forage diets due to limited substrate availability. However, in ruminants fed high levels of starch *Streptococcus* spp. increases lactate production, and negatively impacts the growth of other bacterial species (Jarvis *et al.* 2001; Nagaraja and Titegemeyer, 2007). While the proposed mode of action was supported by the numerically higher lactic acid levels seen in clinically acidotic heifers corresponding with a 4 % increase in *Streptococcus* spp. the persistence of this genus in a variety of diets was not supported. These results indicate a clear correlation between the growth of *Lactobacillus*, *Streptococcus* and an increased severity of response to the acidotic challenge by the individual host. These data were further supported when analyzing the digesta and fluid associated bacterial communities which similarly showed increased levels of *Lactobacillus* and *Streptococcus* as well as *Atopobium, Desulfocurvus, Fervidicola, Lactobacillus, Olsenella, Proteiniborus, RC39, Sharpea* and *Succinivibrio* were all numerically higher in percent abundance during the acidotic challenge. However, other than

Sharpea and *Succinivibrio*, all other genera returned to levels similar to that seen prior to the acidotic challenge. The exact physiological significance of these two genera remains unknown, though they have been described in the gut microbiome of horses, pigs, humans and some ruminants (Bryant *et al.* 1956; Kim *et al.* 2011). By comparing bacterial genera from only clinically and subclinically acidotic heifers based on mean daily minimum pH and pH area below 5.2, this study was able to identify a number of critical bacteria associated with acidic ruminal conditions. Not only did *Streptococcus* spp. and *Lactobacillus* spp. increase, but also did *Selenomonas* spp., *Acetitomaculum, L7A-B08, Pseudobutyrivibrio* and *vadinHA42*. Of these taxa, only unclassified "vadinHA42" has not been previously identified in the rumen, but rather has been associated with the fermentation of wine. Also in this study and previously, it had been noted that *Prevotella* and *Succinivibrio* are responsive to perturbations in the rumen environment such as acidosis (Li *et al.* 2012). Both of these genera increased as the minimum daily pH decreased and the area under pH 5.2 increased. However, due to the ubiquitous presence of *Prevotella* spp. in the rumen as part of the core microbiome, these changes may be strictly due to changes in the availability of nutrients.

Analysis of the post challenge recovery period was done to potentially highlight microbial species, which were either eradicated by an acid imbalance or proliferated and established a sustainable niche within the rumen, thereby permanently changing the dynamics of the microbial population. However, the Challenge Recovery treatment heifers showed no unique species indicative of a modified core taxa post-challenge regardless of whether the samples were epithelial, digesta or liquid associated.

6.2 General Conclusions

The development and advancement of molecular techniques and their use in complex ecosystems such as the rumen has reinitiated investigations into the basic rumen microbial ecology questions from 50 years ago. Mainly, what bacteria species are present in the rumen and what is their role in the rumen ecosystem? Though we are still unable to fully answer these questions, the development of next generation sequencing and renewed sequencing efforts in the Hungate 1000 project ensure that progress is being continually made. To date, limitations inherent in the methodology for classical and molecular microbiology have resulted in incomplete knowledge and many studies have produced conflicting results regarding what species are present at what abundance with changes in diet. However, molecular microbiology has clearly shown that the rumen content associated bacteria are far more diverse than originally ascertained using microscopy and cell counting methods. Analyses of cloned 16S rRNA libraries have indicated that only 11% of the OTUs detected in the rumen by molecular techniques have been previously cultivated in the laboratory. The more recent use of next generation sequencing has further advanced our knowledge of the rumen, showing not only its extraordinary diversity, but also some of the key genes that have made the rumen microbial ecosystem so unique in its ability to convert indigestible feedstuffs into usable energy for the host. However, high numbers of previously unknown/ uncultivated species, combined with sequence databases containing a high number of non-ruminant sequences limits the current capabilities of this technology. It is important to note though, that all modern molecular techniques based on 16S rRNA cannot preclude classic microbiological techniques and they should be used together to ensure accurate results and a detailed analysis of not only the phylogenetic diversity, but also the metabolic capacity of the rumen. Nonetheless, it is evident that uses and development of novel approaches, such as those described above, should continue and be applied more frequently to advance our

understanding of the rumen ecosystem. Furthermore, molecular techniques for rumen ecology offer increased potential to manipulate rumen fermentation to improve ruminant feeding efficiency, and identify perturbations in the ruminal ecosystem such as subacute ruminal acidosis.

The results of this research clearly outline the significance of individual animal variation not only in pH response, but also in microbial profile. Furthermore, these studies were able to clearly establish a rumen core microbiome for the epithelial, solid and liquid associated fractions giving a definitive picture of what bacterial species should be found in all cattle fed a variety of North American diets from grass hay to mixed concentrate rations to finishing feedlot diets. Understanding those species which remain unchanged regardless of nutrient composition in the rumen allows researchers to focus on those species which are sensitive to changes in the environment and those which may be indicative of instability during digestive disturbances such as acidosis. The knowledge provided by this research creates a solid foundation from which to expand our understanding of the rumen ecosystem and how to maximize animal productivity through microbial manipulation.

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APPENDIX A
A.0 INVESTIGATING RUMEN MICROBIAL COMMUNITY STRUCTURE BY MOLECULAR FINGERPRINTING METHODS

A.1 Introduction

Fingerprinting is an affordable, time-efficient molecular analysis that has high-quality repeatability even within complex ecosystems such as the rumen. There are many molecular fingerprinting techniques. However the most commonly used in the rumen are denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment polymorphisms (T-RFLP). While each laboratory has a preferred method of analysis based on experience and available equipment, like any tool for examining the rumen microbial community DGGE and T-RFLP have both positive and negative attributes. Current literature is divided when it comes to determining which technique is most adequate for analysis of complex community samples and no known reports have been published comparing the sensitivity and consistency of these methodologies within the same rumen microbial communities.

Therefore, the objective of this study was to compare DGGE and T-RFLP methods using microbial DNA extracted from the rumen of cattle fed two different diets.

A.2 Materials and Methods

Rumen contents were collected as reported in Chapter 3, section 3.2 from 4 ruminally canulated cattle fed diets containing high concentrate with (HC) or without forage (HCNF) in a randomized cross-over design. Samples were handled anaerobically and separated into liquid and

solid fractions. Microbial DNA was extracted by enzymatic treatment and bead-beating (Chapter 3) and then purified and quantified. The DNA from each sample was separated into duplicate vials for analysis by both DGGE and T-RFLP.

A.2.1 DGGE

As per the description in Chapter 3 (section 3.2), samples were amplified by PCR using the 341F-GC Clamp and 534R primer set (Muyzer *et al.* 1993). Following the protocol in Chapter 3, PCR products were then diluted to equal concentrations and run on a 8% acrylamide gel with a gradient of 45-60% denaturing at 40V for 20 h along with 2 lanes of Nippon Gene II marker (Wako, Nippon Gene, Japan) for calibration within and between gels. Gels were then stained with SybrGold Nucleic Acid Gel Stain (Invitrogen, Life Technologies Corp., Carlsbad, CA, U.S.A) and photographed by UV transillumination.

A.2.2 T-RFLP

Samples were amplified by PCR using 8F (labelled with 6-carboxyfluorescein, 6-FAM) and 926R universal bacterial primers (Brulc *et al.* 2011). The PCR program was set to 95°C for 5 min, with 30 cycles for amplication (95°C, 40 s; 56°C, 30 s; 72°C, 1min) and a final elongation at 72°C for 1 min. Amplification was verified by running PCR products on a 1.5% agarose gel for 20 min at 110V. All samples were then purified using the Qiaquick purification kit (Qiagen, Mississauga, ON, Canada) according to the manufacturers' instructions. Purified DNA (200 ng) from each sample was combined with HaeIII (NEB Inc, Whitby Ontario) for digestion. Digestion of the amplicons was done at 37°C for 4 h. After digestion was complete, 2 µl of each sample was plated into a 96 well plate with 9 µl of Formamide (Hi Di Formamide, Applied BioSystems, California, USA), 0.5 µl of a size standard (Genescan-600 LIZ, Applied BioSystems, California,

USA) and mastermix (Applied BioSystems, California, USA). Plates were analyzed using a T-cycler at 96°C for 5 min to obtain peak profiles.

A.2.3 Statistical Analysis

Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 5.1, Applied Maths, Inc., Austin, TX, U.S.A) and similarity matrices to identify community population differences between treatments, digesta fractions, and individual heifers. Using average Dice's similarity coefficient (D_{sc}) index, with an optimization of 1% and with a tolerance of 1.5%, clustering was carried out using the unweighted pair group method with arithmetic means (UPGMA). Diversity indices were calculated applying the following equations using the band area as determined by BioNumerics software:

Relative Band Area = band area/ Σ(all measured band areas in the sample)

Shannon-Weiner (H′) = Σ (-(Relative Band Area) (Log_{10}(Relative Band Area)))

Simpson's Index (λ) = 1- (Σ Relative Band Area in a Sample)^2

Diversity index values were calculated for each sample and analyzed using the one-way ANOVA procedure of SAS.

A.3 Results

The total number of DGGE bands vs. the number of T-RFLP peaks per dietary treatment differed (P<0.001) between the methods. There were no treatment differences between heifers fed HC vs. the HCNF diet when samples were analyzed using DGGE. However, when samples were analyzed with T-RFLP, heifers fed forage (HC) had less bands than heifers fed no forage (HCNF; Table A1). When fingerprint patterns were compared using cluster analysis, neither

method clustered based on diet, animal or fraction of the digesta contents (solid and liquid; Figure A1).

Using band/peak intensity, fingerprint profiles were analyzed from both the DGGE and T-RFLP analysis for measures of evenness, diversity (Shannon-Weiner) and dominance (Simpsons). Despite variations in the band vs. peak numbers, intensity analysis gave nearly identical results (Table A2). Both analyses showed that the HC diet had significantly higher evenness of the microbial population (DGGE, $P<0.006$; T-RFLP, $P<0.01$), higher diversity (DGGE, $P<0.0001$; T-RFLP, $P<0.0001$), and higher dominance (DGGE, $P<0.0001$; T-RFLP, $P<0.0001$), than the HCNF diet.

Using principle component analysis of peak/band data to explain variance, samples analyzed by DGGE (Figure A2i) and T-RFLP (A2ii) showed completely different special components with only 74% of the variance in DGGE compared to 88% of the variance in T-RFLP being explained by the first three principle components.

Table A1. Effect of diet on number of bands/peaks as determined by DGGE and T-RFLP.

Method	Dietary Treatment	Number of bands/peaks	SE	*P*-value of dietary treatment within method	SE	*P*-value of method
DDGE	HC	28	1.4	0.71	1.05	<0.0001**
	HCNF	29	1.4			
T-RFLP	HC	14	0.77	0.05*	1.22	
	HCNF	16	0.77			

*HCNF has significantly less peaks than HC when using T-RFLP
**DGGE has significantly more bands than T-RFLP has peaks

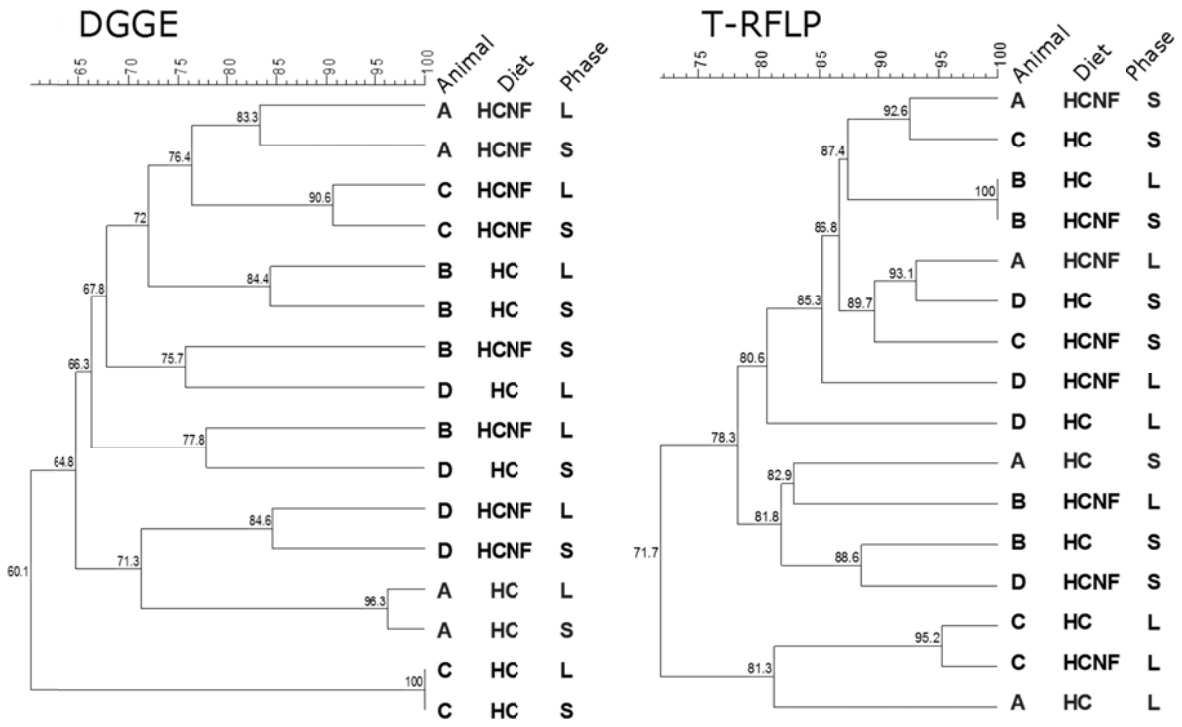
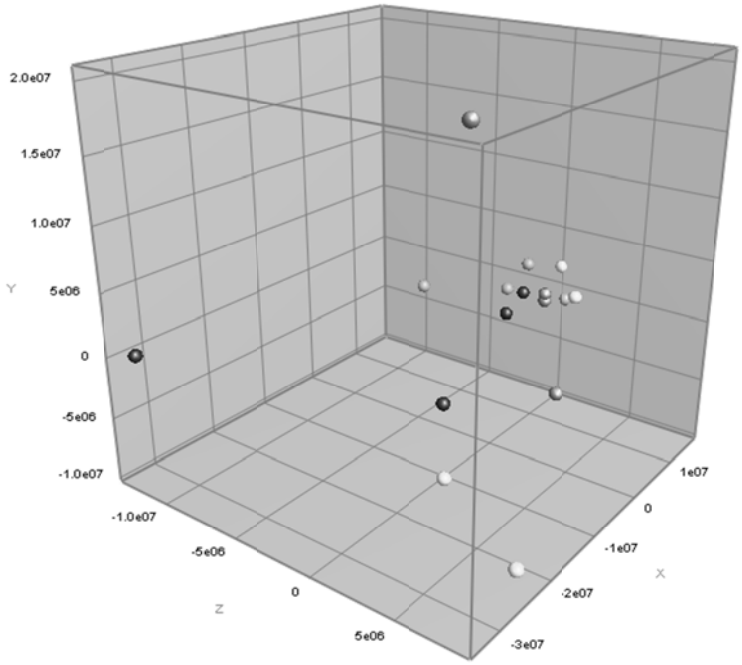


Figure A1. Bionumerics cluster analysis of individual samples analyzed by DGGE and T-RFLP using the presence or absence of bands or peaks

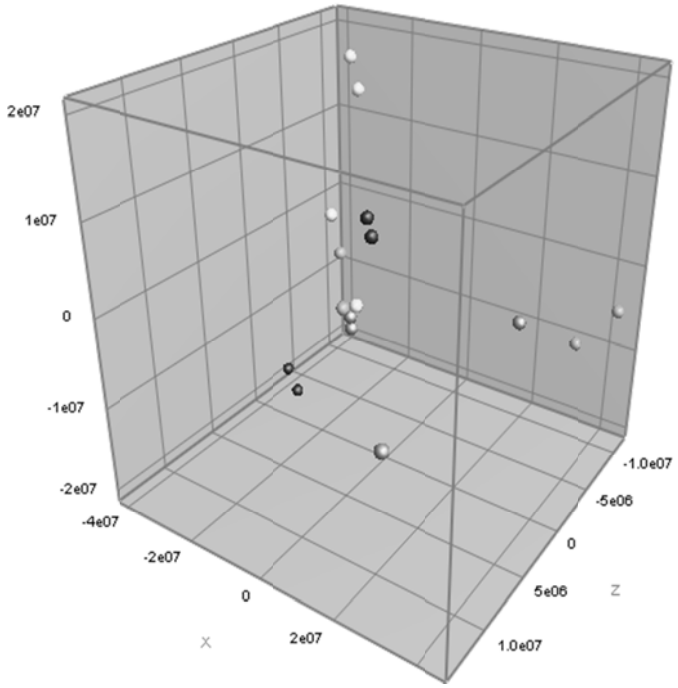
Table A2. Calculated ecological evenness, diversity and dominance indices from T-RFLP and DGGE fingerprint profiles of rumen fluid from high concentrate diets with and without forage.

Method	Dietary Treatment	Evenness	SE	*P*-value	Shannon Weiner Diversity	SE	*P*-value	Simpson's Index Dominance	SE	*P*-value
DDGE	HC	0.86[a]	0.028	0.0056	1.24	0.044	0.0001	0.92a	0.026	0.0001
	HCNF	0.73			0.84			0.24		
T-RFLP	HC	0.91	0.035	0.0098	1.33	0.058	0.0003	0.94	0.034	0.0001
	HCNF	0.77			0.93			0.21		

[a]Mean value under indicated condition
[b]No significant treatment effect was found with either method



A) DGGE



B) T-RFLP

Figure A2. Principle component analysis of peak/band data using 3-D to explaining variance* for samples analyzed by DGGE (A) and T-RFLP (B).

A.4 Discussion

Though many studies have discussed the benefits and pitfalls to fingerprint analysis of complex microbial ecosystems (Muzyer *et al.* 1993; Deng *et al.* 2007), no one has previously compared the use of DGGE and T-RFLP analysis in the same samples obtained from the rumen microbiome. Similar to other procedures, both methods have advantages and disadvantages in describing the bacterial community profile in the rumen. The use of DGGE in the rumen, when the conditions are optimized, can give highly repeatable results (Muyzer *et al.* 1993). Bands from a DGGE gel can also be excised and used for further analysis to define individual ribotypes and give a more accurate diversity analysis. However, DGGE fingerprinting does require significant user input in order to optimize gels and reduce the number of bands which contain multiple bacterial amplicons. While T-RFLP is similarly highly repeatable (Moeseneder *et al.* 1999), the variable use of restriction enzymes can affect peak number and therefore calculation of diversity indices. The variety of restriction enzymes available also makes comparative analyses to other research extremely difficult. Furthermore, the DNA is lost in the process of T-RFLP analysis and therefore identification of peaks as specific ribotypes is not possible.

In this study both microbial fingerprinting methods gave statistically different results although samples were processed following the same procedure and results were analyzed using similar statistical methods. Differences between methods were related directly to the number of peaks or bands which are variable based on analysis. The results of this study suggest a degree of caution should be used when interpreting ribotype community analysis techniques without the support of additional quantitative analysis.